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13482

A Modification of the Diphenylamine Method for Determination of Inulin.

HAROLD E. HARRISON. (Introduced by S. Z. Levine)

From the New York Hospital, and the Department of Pediatrics, Cornell University Medical College.

Colorimetric methods for the determination of inulin in blood serum or plasma and urine based on the reaction of inulin with diphenylamine have been described in recent years. The most widely used procedure is that of Alving, Rubin and Miller,[1] in which the inulin-containing solutions are heated in closed tubes with diphenylamine and hydrochloric acid dissolved in ethyl alcohol. Tightly capped tubes are necessary because of the volatile solvent, and because the solution must be heated for 60 minutes at 100°C to obtain the maximum color. In this laboratory as well as in others, this method has been troublesome and the results not consistently accurate.

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[1] Alving, A. S., Rubin, J., and Miller, B. F., *J. Biol. Chem.*, 1939, **127**, 609.

For this reason a modification has been devised in which a less volatile solvent is used, thus permitting the use of open tubes. With this reagent a shorter period of heating is necessary for maximum color development and accurate results have been obtained in the determination of inulin in blood serum or heparinized plasma in concentrations as low as 4 mg per 100 cc. The determination can be carried out on 0.2 cc.

Method. Reagent. Three grams of diphenylamine, reagent grade, are dissolved in 100 cc of glacial acetic acid and to this solution are added 60 cc of concentrated hydrochloric acid.

This reagent is stable if kept in an amber bottle in a refrigerator at 6°C, and the color production with inulin is found to remain constant for at least one month. A few crystals of diphenylamine may precipitate out of solution but this does not affect the reagent. If kept at room temperature the color in the blank determination becomes progressively darker, but if correction be made for the blank as is automatically done in a photoelectric colorimeter of the Evelyn type the calibration curve is not changed. The color production with different batches of reagent shows only slight variation.

Determination of inulin in blood plasma. It is necessary to remove glucose from the blood plasma since this sugar reacts with the reagent to produce a blue color although to a much less extent than inulin. The glucose is removed by fermentation with washed, starch-free yeast as in the method of Alving *et al.*[1] The plasma proteins are then precipitated by the zinc sulfate method of Somogyi.[2] The protein-free filtrate is diluted so that the final solution contains between 2 and 12 μg of inulin per cc. For most determinations the micro precipitation technique of Somogyi in which the filtrate represents a 1 :40 dilution of the plasma will be found suitable.

Two cc of the filtrate are measured into a pyrex test tube, 150 by 16 mm. Four cc of the diphenylamine reagent are accurately pipetted into the tube. (The reagent is drawn into the pipette with a rubber bulb.) The tube is agitated to mix the solutions thoroughly and is then placed in a rack in a boiling water bath. The bath should be adjusted so that the rate of boiling is uniform and the level of water is kept constant at about the level of the solution within the tube. A blank, containing 2 cc of distilled water and 4 cc of reagent is heated simultaneously. After 30 minutes the tubes are removed from the boiling water and cooled to room temperature. The color is then quantitatively measured in a photoelectric colorimeter using a light filter with maximum transmission at 620 millimicrons. We have used the Evelyn photoelectric colorimeter with the aperture set at 6 cc and the 620 filter. For the most accurate results it is advisable to run a blank determination on a sample of serum or plasma obtained before the administration of inulin. This blank value is small, if freshly prepared yeast suspensions are used, and does not vary widely with different samples of serum or plasma so that an average blank value may be determined for several samples and this value used thereafter. The average blank value for a group of human sera was found to be equivalent to 1 mg inulin/100 cc serum.

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[2] Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

The method is calibrated by treating known solutions of inulin with the diphenylamine reagent. The color formation follows the Lambert-Beer law as indicated by the linear relationship between the concentration of inulin and the logarithm of the light transmission.

Determination of inulin in urine. Normal urine contains solutes which react with diphenylamine to give a blue color. The blank values obtained vary with the degree to which the urine is concentrated by the kidney. Samples of normal human urine excreted at the rate of approximately 1 cc per minute were found to give a color equivalent to 10 mg of inulin per 100 cc of urine. In the determination of renal clearances of inulin the concentrations of inulin in the urine are so great that the urine corresponding to an excretion rate of 1 cc per minute may be diluted to 1:1000 or more and at these dilutions the blank values are so small that they may be disregarded. If glucose is present it must be removed by fermentation. Moreover, if the urine contains significant amounts of protein, deproteinization must be carried out as in the plasma method. Ordinarily, however, it is only necessary to dilute the urine so that

TABLE I.
Recovery of Added Inulin.

	Inulin added, mg/100 cc	Inulin recovered, mg/100 cc	%
Serum	3.2	3.25	101.5
	4.0	3.68	92.0
	4.0	3.95	98.6
	8.0	8.05	100.6
	20.0	19.4	97.0
	20.0	20.2	102.0
	33.3	33.7	101.2
	33.3	33.1	99.4
	33.3	33.0	99.1
Urine	100	100	100
	200	196	98
	500	500	100
	500	510	102

the concentration of inulin is between 2 and 12 μg per cc. Two cc of the diluted urine are measured into a test tube, 4 cc of the reagent added and the tube heated in a boiling water bath as previously described for plasma.

In Table I are shown the results of recovery experiments. Measured amounts of inulin were added to blood serum and the removal of glucose and protein carried out as described. Blank determinations were made on each sample of serum. In the case of the urine, the inulin was added in the concentrations shown, and the determinations made directly following suitable dilution. The urine specimen was a highly concentrated one and the blank value was equivalent to 51 mg inulin per 100 cc of undiluted urine.

13483 P

The Nervous Factor in Burns.

HERMAN KABAT* AND RAYMOND F. HEDIN.
(Introduced by M. B. Visscher.)

From the Interstate Clinic, Red Wing, Minn., the Anderson Institute for Biological Research, and the Department of Physiology, University of Minnesota.

While evidence has been accumulating concerning the importance of a nervous factor in the etiology of traumatic shock,[1-3] similar studies of the problem of burns have not been carried out.

A comparison was made of the effects of thermal trauma to one hind leg in control cats and in spinal animals. Transection of the spinal cord at L_1 or T_{11} effectively eliminated the influence of afferent nerve impulses from the burned leg but left the major vasoconstrictor outflow from the spinal cord intact.

The experiments were performed on 83 adult cats, anesthetized throughout the procedure with pentobarbital sodium. One hind leg was burned with a Bunsen flame for 10 or 15 minutes. The spinal cord was transected about 30 minutes preceding application of thermal trauma. Observations were made of blood pressure (carotid cannula), hemoglobin and specific gravity of whole venous blood,

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* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] O'Shaughnessy, L., and Slome, D., *Brit. J. Surg.*, 1935, **22**, 589.

[2] Lorber, V., Kabat, H., and Welte, E. J., *Surg. Gyn. and Obst.*, 1940, **71**, 469.

[3] Freedman, A. M., and Kabat, H., *Am. J. Physiol.*, 1940, **130**, 620.

(falling drop technique), respiration, body temperature, survival, local fluid loss in the burned limb and pathology.

In control cats, burning resulted in an abrupt rise in arterial pressure from 120 to 160 mm Hg. The blood pressure remained high and only reached the original level after 10 hours. On the other hand, arterial pressure fell sharply in spinal cats during thermal trauma from an initial 90 mm Hg to 60 mm Hg; it was restored in the course of 1.5 hours and then maintained between 100 and 80 mm Hg. While arterial pressure fell quickly to 90 mm Hg as a result of spinal transection alone, without burning it remained at about this level for many hours. The rise in blood pressure from burns in control cats is thus apparently reflex and masks an initial sharp fall due to non-nervous factors.

Of greatest interest was the study of blood concentration by determination of hemoglobin and specific gravity. In control cats, hemoglobin determinations showed that the blood had concentrated 34.5% 30 minutes after the burning and attained a maximum concentration of 44% at 8 hours. Burns in spinal cats resulted in a hemoconcentration of only 14.5% at 30 minutes and a maximum of 32% at 7 hours. Measurement of blood specific gravity gave similar results. In control cats, specific gravity increased 0.0106 20 minutes after the burn and reached a maximum increase of 0.0127. In contrast to this, burns in spinal cats caused an increased specific gravity of only 0.0027 at 20 minutes and a maximum rise of 0.0066. Thus, elimination of the nervous factor decreases the rate and maximum extent of hemoconcentration following burns.

Respiratory rate increased markedly as the result of thermal trauma but since the rise was greater in spinal animals than in controls, the increased rate of respiration cannot be attributed to the nervous factor.

Great variations were noted in survival time following apparently identical thermal trauma. In 24 control cats, survival ranged from 1.5 to 80 hours, with the average 32.5 hours. In 19 spinal cats, average survival following the burn was 40 hours, with a range from 10 to 75 hours. No conclusion can be drawn concerning survival because of the wide range of variation of the individual experiments.

Local fluid loss was measured by comparing the weights of the normal and burned hind quarters. Local fluid loss averaged 2.5% of the body weight in controls and 1.5% in spinal cats.

Gross pathology was similar in incidence and severity in control and spinal animals. Most of the cats showed pulmonary and renal congestion and dark bloody bladder urine. Other frequent lesions were subendocardial hemorrhages in the left ventricle, and acute hemorrhage or ulceration in the pylorus or duodenum. Pleural effusion, ascites, congestion and edema of the intestines were never observed and pericardial effusion was very infrequent.

The decrease in hemoconcentration resulting from removal of the nervous factor may perhaps be accounted for on the basis of elimination of the reflex rise in arterial pressure, contraction of the spleen and vasoconstriction. Filtration of fluid into the burned skin is apparently decreased by functional deafferentation.

13484 P

On Infectious Mononucleosis.

A. NETTLESHIP. (Introduced by E. M. K. Geiling.)

From the Montgomery County Laboratories, Amsterdam, N.Y.

In the spring of 1941, during the occurrence of an epidemic of infectious mononucleosis, we attempted to isolate a transmissible agent from sterile Berkefeld filtrates of nasal washings and whole blood by inoculation into the chorio-allantoic membrane of the chick embryo. Material was obtained from patients who gave a characteristic history, blood picture, and whose heterophile antibody titers ranged from complete agglutination of 160 to a high of 2560. We also attempted passage from patients whose clinical history and leucocyte picture were typical and who later gave heterophile agglutinations in the range mentioned. The nasal washings and blood from some patients were frozen for 12 hours before they were used. Two additional cases made their appearance this fall. In all, a total of 8 attempts was made to transmit the agent from 8 cases; positive results were obtained from 4. The sterile filtrate, when inoculated onto the membrane, showed small pearl-gray reactions with a spreading cloudiness about them. This reaction occurred after the third to the fifth day of inoculation and continued to grow until the membranes were harvested for study. Of 66 membranes which were inoculated with sterile nasal washings, 19 showed positive reactions. Of 30 membranes inoculated with whole blood, 13 showed positive reactions. Cultures from all infective membranes were bacteriologically sterile. Nasal washings and blood from 2 normal individuals were negative. Microscopically, the positive membrane showed an ectodermal proliferation and heavy monocytic cell infiltration. Although special stain was applied, (Mallory phloxine-methylene blue) no inclusion bodies were found. We were able to continue the reaction through 4 to 14 passages obtaining positive results with about 50% of the membranes. The agent finally died out, showing a dramatic drop of positive takes in the last 2 generations of each passage. An attempt to transmit the virus into 25 mice through the intraperitoneal route was unsuccessful. While we could reproduce a monocytosis (prolymphocyte) in rabbits, by injection of a suspension of infective ground chick membranes we were unable to get a heterophile reaction of significantly high titer against sheep cells with the rabbit serum. Serum from normal rabbits did not give a positive heterophile reaction against sheep cells. The leucocyte picture in rabbits made its appearance 4 to 6 weeks after inoculation.

There have been attempts to prove the relationship of various bacteria to infectious mononucleosis. The only trials which have been made, insofar as we could determine from the literature, with the use of bacteria-free filtrates were those by van den Berghe and Liessens.[1, 2] They consider the agent a virus with which, after primary isolation on tissue culture, they were able to reproduce the disease in monkeys. They obtained a typical blood picture and heterophile reaction. A suggestion of theirs[3] that the virus survives at a low temperature prompted our freezing the material prior to inoculation on the membrane. This seemed to increase its infectibility.

I wish to thank J. Gray for technical assistance.

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[1] van den Berghe, L., and Liessens, P., *Compt. rend. Soc. de Biol.*, 1939, **130**, 279.

[2] van den Berghe, L., and Liessens, P., *Compt. rend. Soc. de Biol.*, 1939, **131**, 156.

[3] van den Berghe, L., and Liessens, P., *Compt. rend. Soc. de Biol.*, 1939, **132**, 90.

13485

Effect of Parenteral Amino Acids (Hydrolyzed Casein) on Growth of Rats.*

ABRAHAM HORVITZ, LEO A. SACHAR AND ROBERT ELMAN.
(Introduced by Evarts A. Graham.)

From the Department of Surgery, Washington University School of Medicine, and Barnes Hospital, St. Louis, Mo.

Humans and experimental animals may be placed in positive nitrogen balance by the intravenous administration of a protein hydrolysate.[1-3] Moreover, casein digests given parenterally are utilized for the manufacture of serum proteins in dogs made hypoproteinemic by dietary means,[4] or by plasmapheresis.[5] Experiments are presented herein showing that growth is promoted when protein hydrolysates are injected subcutaneously as the sole source of nitrogen in the diet (with the exception of very small amounts in the vitamin supplements).

Methods. The hydrolysate used (Amigen) was prepared by Mead Johnson and Company, it is an enzymatic digest of casein and pork pancreas, containing two-thirds amino acids and the rest in the form of dipeptides. Five per cent solutions of Amigen were made in distilled water and the pH adjusted to 7.4 with sodium hydroxide. The solutions were then sterilized by passing them through a Berkefeld filter. When given parenterally the solution was injected slowly into the subcutaneous tissues of the back of the rat through a 31 gauge needle.

The non-protein (basal) portion of the diet was given by mouth. It is described by Miller, Kemmerer, Cox and Barnes,[6] and was kindly furnished by them. It was shown by these authors not to support growth; however, they found good growth occurred when 15% Amigen was added thereto.

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* Aided by grants from the Louis D. Beaumont Fund and Mead Johnson and Company.

[1] Henriques, V., and Andersen, A. C., *A. f. physiol. chemie,* 1913, **88**, 357.

[2] Elman, R., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 610; *Ann. Surg.*, 1940, **112**, 594.

[3] Shohl, A. F., Butler, A. M., Blackfan, K. D., and MacLachlan, E., *J. Pediat.*, 1939, **15**, 469.

[4] Elman, R., Sachar, L. A., Horvitz, A., and Wolf, H., *Arch. Surg.*, in press.

[5] Madden, S. C., Zeldis, L. J., Hengerer, A. D., Miller, L. L., Rowe, A. P., Turner, A. P., and Whipple, G. H., *J. Exp. Med.*, 1941, **73**, 727.

[6] Mueller, A. J., Kemmerer, K. S., Cax, W. M., Jr., and Barnes, S. T., *J. Biol. Chem.*, 1940, **134**, 573.

Newly weaned rats of the Anheuser-Busch strain were used; the duration of each experiment was 7 days. The amount of solution injected each day was 12 to 16 cc (0.6 to 0.8 g of Amigen) and was divided into 5 equal doses given 1½ to 2 hours apart. Weights were taken each morning just before the first injection, *i.e.*, 10 to 12 hours after the last injection of the previous day. No edema was noted after this interval.

Experiment I. The rats in this experiment were divided into 3 groups. Group A consisted of 3 rats which received the stated dose of Amigen parenterally, Group B of 5 rats which received the same amount of Amigen by mouth. Each group was offered the basal diet in amount to make up 85% of the total diet. Group B consumed all of its food invariably, in contrast to the injected rats, which ate very little of their basal diet. Thus Groups A and B received the same amount of Amigen, but varying amounts of the basal diet. Group C received the basal diet alone.

Experiment II. The rats in this experiment were divided into 2 groups. Group A was composed of 2 rats which received the stated dose of Amigen parenterally, Group B, of 3 rats, which received in the same manner and in similar amount a 5% solution of glucose in physiological saline solution. Both groups were offered the basal diet by mouth ad libitum. As in Group A of Experiment I the injected rats of both groups failed to consume all of their basal diet. The purpose of this experiment was to determine the effect of injected fluid itself upon the weight of the rats, *i.e.* to rule out the possibility that any gain in weight might be due to subcutaneous edema produced by the injection.

Experiment III. In this experiment, the method of paired feedings was used in order to remove differences due to variations in the amount of basal diet consumed. Two groups of rats were used; both received the stated amount of Amigen, Group A parenterally, Group B by mouth. In Group B the same amount of basal diet was fed by mouth as the injected rats of Group A had consumed the previous day. There were 4 rats in the first group and 2 in the second group. Weight changes on the day in which the rats had the same diet were then compared. In this experiment, the daily parenteral dose was divided into only 3, instead of 5, injections.

Findings and comment. The results (shown graphically in the figure) indicate that some growth may be achieved on a diet, the sole source of nitrogen of which is given parenterally in the form of a solution of hydrolyzed protein. Thus, in 3 experiments, each for a period of 7 days, gains in weight (as measured from the time in-

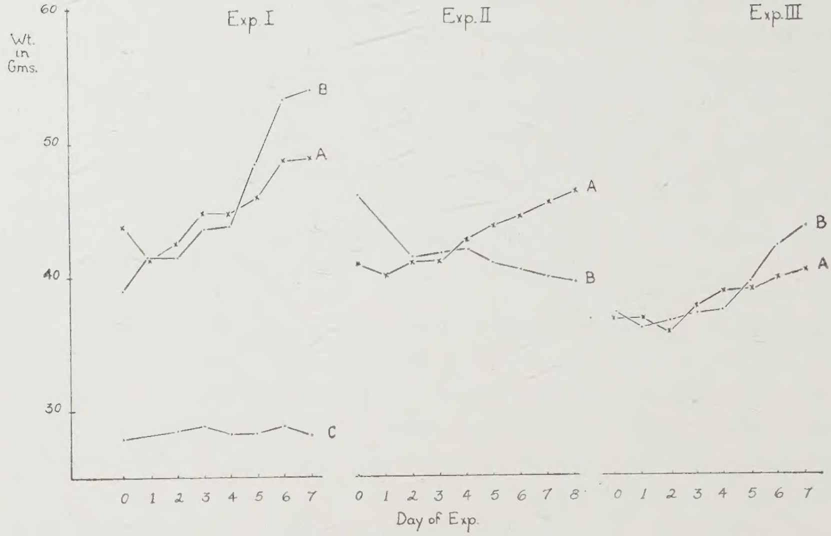

FIG. 1.

jections were begun to the time they were discontinued) were obtained, varying from 9 to 11% (Curves A). While it is true that rats receiving Amigen by mouth in Experiment I, (Curve B) gained more in weight, this was probably due to the fact that they consumed more of the non-protein part of the diet than the injected animals. It is noteworthy that even for the small amounts of Amigen injected, a large amount of fluid is required as a vehicle; in many instances, the daily volume equalled approximately half of the body weight. This of itself may have so influenced the animals that they failed to consume the whole of their allotted basal diet. The problem of hypodermoclysis is due to the necessity of giving the solution in an isotonic form, thus requiring large volumes of fluid to make up the large amount of protein needed for growth in rats. For example, to give what would be an adequate protein intake (3 g daily), 60 cc of solution would have to be injected, or nearly twice body weight of the rats. The discrepancy in the amount of basal diet consumed was controlled in the paired feeding experiment performed in Experiment III; the growth curves were similar, whether the Amigen was given by mouth or parenterally, although actually not very different from the uncontrolled Experiment I. The failure of rats injected with glucose in saline to gain weight, the absence of clinically obvious edema, and the long interval which was allowed to elapse between the last injection and the taking of the weight all indicate that the observed gain in weight was due to growth and not to accumulation of fluid.

Summary. Growth of rats was obtained when the sole source of nitrogen in the diet was provided by parenterally injected casein digest.

13486 P

Development of Tolerance to Gold Salts in Rats.

RUTH CORTELL AND R. KOHN RICHARDS.

From the Abbott Laboratories, North Chicago, Ill.

The minimum lethal dose of gold sodium thiosulphate intramuscularly in rats was found to be approximately 35 mg/kg. The rats which survived this dose were given repeated doses of 35 mg/kg every other day and then tested for the maximum tolerated dose of gold sodium thiosulphate, as compared to normal rats (Table I).

TABLE I.
Tolerated Doses of Gold Sodium Thiosulphate in Normal and Tolerant Rats.

	Normal rats		Tolerant rats*	
Dose, mg/kg	Survived, No.	Fatal, No.	Survived, No.	Fatal, No.
25	7	0		
40	1	3		
50	1	21		
100	1	31	7	0
150			3	0
200			0	3
250			0	3
300			0	2

*This group received 4 doses of 35 mg/kg every other day and was tested on the second day after the last chronic dose.

Whereas normal rats tolerated less than 40 mg/kg, animals that had received 35 mg/kg of gold sodium thiosulphate intramuscularly every other day for 4 doses tolerated 150 mg/kg, an increase of over 200%.

The relation of the size and number of doses to the development of tolerance is shown in Table II. The criterion of tolerance formation was survival following the injection of 100 mg/kg of gold sodium thiosulphate, a dose that is practically invariably fatal in normal rats. 35 mg/kg were given every other day to 36 rats, and the test dose of 100 mg/kg was given after a varying number of doses. As few as 2 chronic doses were found to be sufficient to cause the formation of tolerance to gold sodium thiosulphate, although not

TABLE II.
Development of Tolerance to Gold Sodium Thiosulphate in Rats.

Dose, mg/kg	No. of doses*	Days between last chronic dose and test dose (100 mg per kg) †	Rats survived, No.	Rats fatal, No.
35	13	2	3	0
	10	2	7	0
	5	2	3	0
	5	16	1	2
	4	2	4	0
	4	4	3	0
	4	8	3	0
	4	16	1	1
	2	2	5	1
	2	16	2	0
20	5	2	3	0
	8	2	2	0
10	5	2	0	2
	8		0	3

*Doses given every other day.

†30 out of 31 normal rats died on 100 mg/kg of gold sodium thiosulphate intramuscularly.

in all of the rats. Four doses produced tolerance in all the rats. Rats were tested not only on the second day after the last chronic injection, but also at varying intervals after this, up to the sixteenth day. On the eighth day the tolerance was still present, but on the sixteenth day, the tolerance had begun to disappear, even in rats that had received a larger number of doses.

Smaller doses than the maximum tolerated dose of gold sodium thiosulphate were found capable of producing tolerance, as for example, 20 mg/kg, but reduction of the dose to lower levels resulted in no tolerance formation.

Normal rats were found to tolerate 74 mg/kg of gold sodium thiomalate. Six rats given 55 mg/kg of gold sodium thiomalate intramuscularly every other day for 8 doses were tested with 185 mg/kg of gold sodium thiomalate, a dose invariably fatal in normal rats. All of the rats survived. Thus, it was demonstrated that tolerance could be formed to gold sodium thiomalate as well as to gold sodium thiosulphate. Three rats given chronic doses of gold sodium thiomalate survived 100 mg/kg of gold sodium thiosulphate, and similarly, 3 rats given chronic doses of gold sodium thiosulphate, survived 185 mg/kg of gold sodium thiomalate, revealing a cross tolerance between the two drugs.

Summary. Tolerance to both gold sodium thiosulphate and to gold sodium thiomalate can be developed in rats, as well as a cross tolerance between the 2 compounds. A minimum dose is necessary for tolerance formation, but with a sufficient dose, tolerance can be developed with only 2 doses. The tolerance lasts for considerable time.

13487

Distribution of Body Water Following Hemorrhage.

A. M. LANDS AND WALTER JOHNSON.

From the Department of Physiology, Schools of Medicine and Dentistry, Georgetown University, Washington, D.C.

Circulating blood increases its water content following hemorrhage.[1] This dilution is generally believed to result from the passage of non-vascular extracellular water into the vascular space due to a reduced capillary pressure following a reduction in blood volume. On the other hand, a few investigations as those of Milroy,[2] Gamble, Ross and Tisdall,[3] Kerr,[4] and Stewart and Rourke[5] have suggested the possibility that cellular fluids may be drawn upon to replace a deficit in blood volume.

The development of practical methods whereby the volume of extracellular water may be determined (Crandall and Anderson,[6] Gregerson and Stewart,[7] Brodie and Friedman,[8] Wallace and Brodie[9]) has suggested the desirability of applying these technics in an attempt to determine the changes in the volume of cellular and extracellular water that take place following hemorrhage. This was attempted by measuring the volume of sulfocyanide available water both of the whole animal and of some of its organs after hemorrhage and by comparing these values with those obtained from normal animals.

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[1] Adolph, E. W., Gerbasi, M. J., and Lepore, M. J., *Am. J. Physiol.*, 1933, **104**, 502.

[2] Milroy, T. H., *J. Physiol.*, 1917, **51**, 259.

[3] Gamble, J. L., Ross, G. S., and Tisdall, F. F., *J. Biol. Chem.*, 1923, **57**, 633.

[4] Kerr, S. E., *J. Biol. Chem.*, 1926, **67**, 689.

[5] Stewart, J. D., and Rourke, G. M., *J. Clin. Invest.*, 1936, **15**, 697.

[6] Crandall, L. A., and Anderson, M. X., *Am. J. Digest. Dis. Nutrition*, 1934, **1**, 126.

[7] Gregersen, M. I., and Stewart, J. D., *Am. J. Physiol.*, 1939, **125**, 142.

[8] Brodie, B. B., and Friedman, M. M., *J. Biol. Chem.*, 1937, **120**, 511; 1939, **130**, 555.

[9] Wallace, S. B., and Brodie, B. B., *J. Pharmacol. and Exp. Therap.*, 1939, **65**, 214.

Methods. Cats anesthetized with pentobarbital sodium (40 mg per kilo of body weight) were used throughout. In 7 experiments the spleen was left intact. In 8 others, the spleen was carefully removed through an incision in the lateral abdominal wall without appreciable loss of blood. A known amount of sodium sulfocyanide (as a 5% solution to make about 200 mg per kg of body weight) was injected into the circulation by way of the femoral vein, 30 to 60 minutes being allowed for its distribution. The carotid artery was then cannulated and about 10 ml of blood removed for the various analyses. Following this, the artery was again opened and the cat bled until a total of 15.2 to 26.1 ml of blood per kg of body weight had been removed. Sixty to 120 minutes later, a final sample of blood was taken for analysis and the animal sacrificed by asphyxia. Various organs were removed, carefully blotted to remove excess blood, minced, weighed and dried for 24 to 48 hours in an oven maintained at 105°C. The dry tissues were then dissolved in normal KOH and analyzed for sulfocyanide.[8] Normal control values used for comparison in these experiments are those of Lands, Cutting and Larson[10] uncorrected for fat content. Interfering colors make sulfocyanide analysis of the liver difficult. For that reason the extracellular water is here computed from its chloride content. The chloride was determined by the method of Sunderman and Williams.[11] Methods of computation are described in an earlier publication.[10] Excretion of water and injected sulfocyanide was so small under the conditions of these experiments that this can be disregarded.

The following analyses were made on blood; specific gravity of whole blood and serum,[12] serum chloride,[13] serum sulfocyanide,[6] and serum potassium.[14, 15]

Results. Following hemorrhage, the total volume of water available for the solution of sulfocyanide was increased 34.9 to 121.0 ml per kg of final body weight (Table I). This increase was associated with a reduction in the chloride concentration of serum water in all experiments but one. For example, in Experiment I (Table II) the chloride concentration of serum water was reduced

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[10] Lands, A. M., Cutting, R. A., and Larson, P. S., *Am. J. Physiol.*, 1940, **130**, 421.

[11] Sunderman, F. W., and Williams, P. J., *J. Biol. Chem.*, 1933, **102**, 279.

[12] Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

[13] Van Slyke, D. D., *J. Biol. Chem.*, 1923, **58**, 523.

[14] Shohl, A. T., and Bennett, H. B., *J. Biol. Chem.*, 1928, **78**, 643.

[15] Hartzler, Eva R., *J. Biol. Chem.*, 1937, **122**, 19.

TABLE I.

Change in Total Volume of Sulfocyanide Available Water Following Hemorrhage.

Exp. No.	Sulfocyanide available H_2O, ml/kg	Hemorrhage, ml/kg	Time elapsed after hemorrhage, min.	Final sulfocyanide available H_2O, ml/kg final body wt	Inc. sulfocyanide available H_2O, ml/kg final body wt
1.	234	24.0	60	342	121.0
2.	169	15.2	60	247	81.6
3.	204	16.1	60	263	67.6
4.	300	17.7	70	330	34.9
5.	288	26.1	80	354	71.0
6.	261	16.5	150	317	58.5
7.	242	20.9	90	320	85.6
9.*	154	a. 11.2	a. 10	179	29.9
		b. 17.5	b. 40	207	39.4
10.	358	0.0	290	358	0.0
11.	303	0.0	285	303	0.0

*Not splenectomized.

TABLE II.

Changes in Serum Chloride Concentration Following Hemorrhage. All values in mM/kg serum water.

Exp. No.	Before hemorrhage	After hemorrhage	Conc. in water added
1.	127	109	74
2.	141	130	122
3.	123	105	53
4.	113	119	175
5.	122	114	46
6.	125	118	107
7.	125	120	93
8.	118	113	98
9*	144	129	71

*Not splenectomized.

Serum protein values determined from the specific gravity according to the formula $P = 345(G - 1.0076)$; Kagan.[20]

from 127.0 mM to 109 mM per kg whereas at the same time there was an increase of 121.0 ml per kg of body weight in the sulfocyanide available water. In 3 experiments serum analysis for potassium indicated a definite increase. Before hemorrhage values for serum potassium were 6.7, 5.1 and 6.1 mM per kg of serum water; after hemorrhage 12.1, 9.35 and 14.1 mM per kg of serum water.

Analyses of skeletal muscle for changes in total water and for sulfocyanide available water gave results which suggest no significant change in the distribution of water in this organ following hemorrhage. In one instance there was found an increase in total water of 30 ml per 100 g of solids and in 2 instances a decrease of 22 ml per 100 g of solids (Table III). Similar results were obtained

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[20] Kagan, Benj. M., *J. Clin. Invest.*, 1938, **17**, 369.

TABLE III.
Change in Total Water Content Following Hemorrhage.
(All values in ml/100 g solids.)

Exp. No.	Gastrocnemius muscle	Diaphragm muscle	Cardiac muscle	Liver	Pancreas	Lung	Pylorus	Duodenum	Colon	Skin
1.	6	—4	9	—13	264	22	77	50		55
2.	30*									92*
3.			32	41	28	11	51	6	17	10*
4.	— 8*									14*
5.	—22*		49	17	101	—4		70	80	—22*
9.	—22	16	11		23		65	18	—4	5
A.	26	11		14	231		77	32	21	93
B.	8	—24	45	36	108		72	32	40	25
C.	19	—63	52	52	91	3	50	0	16	—17
D.	10	12	68	41	41	70	85	13	69	28
E.	— 5	—61	52	14	55	10	57		8	22

*Control tissue taken from same experimental animal. In all other instances the differences are based on the normal averages. (12 experiments uncorrected for fat; Lands, Cutting and Larson, 1940.)

TABLE IV.
Changes in Sulfocyanide Available Water Following Hemorrhage.
(All values in ml/100 g solids.)

Exp. No.	Gastrocnemius muscle	Diaphragm muscle	Cardiac muscle	Liver†	Pancreas	Pylorus	Duodenum	Colon	Skin
1.	25.8	8		50	93	112	91	100	96
2.	— 9.0*								74*
3.	4.0	—40	10		29	78	22	10	9*
4.									35*
5.	33.0*		40	18		88	2	—9	2*
6.	— 1.2	— 7	59		3	122	52	143	18
A.	—10.3	8		18	255	11	0	47	—9
C.	—21.7	28	59	34	5	66	171	101	7
D.	35.8	64		31	73	157	103	97	58
E.	24.0		—36	14	123	42	—62	—96	38

*See footnote, Table III.
†No sulfocyanide analyses could be made on liver due to interfering colors. The values given here are those for chloride available water.

for diaphragm. In cardiac muscle, liver, pancreas, lung, pylorus, duodenum, colon and skin significant increases were found in the total water content and in the sulfocyanide available water content (Tables III and IV). This was considerable for the pancreas and for the alimentary tract. In general, when the total extracellular compartment was increased most there were large increases in both the total water and in the sulfocyanide available water of the pancreas and alimentary tract.

Discussion. The experimental findings presented here are inconsistent with the simple concept which attributes blood dilution to the movement of a lymph-like fluid from the interstitial spaces into the blood stream. A reduction of body water by hemorrhage should lead to a corresponding reduction in the sulfocyanide available (extracellular) water. Quite the opposite was found to be the case, the sulfocyanide available water being increased after hemorrhage. The results of the experiments here reported suggest the increase in sulfocyanide available water comes from either (1) an increase in the permeability of some of the tissue cells to sulfocyanide following hemorrhage or (2) an increase in the volume of extra-cellular water at the expense of cell water. Inasmuch as the total water as well as the sulfocyanide available water increased in some of the organs (as intestine, pancreas and other visceral organs) it would seem more plausible to assume that the increase in the volume through which the sulfocyanide is distributed actually represents an increase in the total extracellular water at the expense of the water content of some organs of the body. The possibility that the cellular compartment may lose water after hemorrhage had been suggested as early as 1917 when Milroy[2] reported, on the basis of changes in the specific gravity of both whole blood and serum and a diminished alkaline reserve of plasma, that a hypotonic tissue fluid low in bicarbonate entered the blood stream. Johnston and Wilson[16] likewise found a reduced alkaline reserve after hemorrhage. Gamble, Ross and Tisdall,[3] Gamble[17] and Butler, McKhann and Gamble[18] have shown that tissue fluid may be drawn upon to replace extracellular water and that this water is made available through the destruction of protoplasm. Kaump and Parsons[19] report an increase in the concentration of blood urea nitrogen following hemorrhage, this increase being at its maximum 24 to 48 hours following hemorrhage when blood dilution is greatest. This would be expected to follow if cell water is liberated through the destruction of protoplasm. Kerr[4] found the serum sodium and chloride diminished without a reduction in the concentration of serum potassium following hemorrhage. This, he believed indicated a mobilization of intracellular water relatively rich in potassium and low in sodium and chlorides. In 3 experiments, in which serum potassium was determined, we found potassium elevated to a value about twice the normal. Careful analysis of skeletal muscle failed to demonstrate any significant change in either its total water content or its sulfocyanide available water content. This organ, the most probable source of available water because of its large mass, could have given up water to the extracellular compartment only by a reduction of its mass of protoplasm.

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[16] Johnston, C. G., and Wilson, D. W., *J. Biol. Chem.*, 1929-30, **85**, 727.

[17] Gamble, J. L., Ross, G. S., and Tisdall, F. F., *J. Biol. Chem.*, 1923, **57**, 633.

[18] Butler, A. M., McKhann, C. F., and Gamble, J. L., *J. Pediat.*, 1933, **3**, 84.

[19] Kaump, D. H., and Parsons, J. C., *Am. J. Digest. Dis. Nutrition*, 1940, **7**, 191.

Summary. 1. The volume of sulfocyanide available water of cats is definitely increased after hemorrhage. 2. The chloride concentration of serum water was found to be decreased in all experiments save one. 3. The total water and sulfocyanide available water content of cardiac muscle, pancreas, pylorus, duodenum, colon, skin and liver (chloride available water) was increased after hemorrhage. No significant differences were found in the case of skeletal muscle (gastrocnemius and diaphragm). 4. The significance of the above findings is discussed.

13488

A Simple Method for Prolonging Therapeutic Insulin Coma.*

JOSEPH WORTIS AND IRVIN M. KORR. (Introduced by Homer W. Smith.)

From Bellevue Psychiatric Hospital and New York University College of Medicine, New York City.

In the treatment of psychoses, insulin coma[1] (or the period of unconsciousness) is generally not allowed to persist beyond an hour or an hour and a half. In cases inadvertently allowed to linger in coma beyond that period, an irreversible insulin coma[2] frequently follows that can no longer be relieved by intravenous glucose. This dangerous and sometimes fatal complication has, however, directed the attention of several investigators to the important fact that patients who survive several hours or days of this relatively irreversible coma often show dramatic psychiatric improvement on awakening. Kraulis[3] attempted to utilize this phenomenon for therapeutic ends by prolonging insulin coma with periodic small glucose feedings sufficient to sustain the patient but not sufficient to rouse him. The difficulties in the clinical management of these sporadic feedings, however, and the frequent presence of gastric retention[2] (which

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* This work was aided by the Havelock Ellis Fund for Psychiatric Research.

[1] Sakel, Manfred, *Insulin Shock Treatment of Schizophrenia,* Nervous and Mental Disease Monograph No. 62, New York, 1937.

[2] Wortis, Joseph, and Lambert, R. H., *Am. J. Psychiatry,* 1939, **96**, 335.

[3] Kraulis, W., *Schweiz. Arch. f. Neur. u. Psychiat.,* Erganzungsheft, 1937, **39**, 219.

Kraulis did not suspect) made this mode of treatment too dangerous for clinical application.

It can be assumed that the cerebral injury which ensues in accidentally protracted coma is due to the diminution of brain metabolism (by withdrawal of substrate) below levels adequate for cell maintenance over long periods of time. In our cases (30 experiments on 10 subjects), we have succeeded in prolonging hypoglycemia as long as 21 hours and coma as long as 18 hours, without mishap, by sustaining brain metabolism above this dangerous level, but still below levels which support consciousness and other associated cerebral functions. This has been achieved by administering infusions of 5% glucose in physiological saline (Sterisol) intravenously at controlled rates. Inasmuch as large doses of insulin exert their hypoglycemic effect for many hours after administration,[4] one need only maintain the blood glucose at a moderately high level (*e.g.,* 35 mg per 100 ml) to prolong coma for many hours. When the insulin effect begins to subside, a fresh intramuscular injection of insulin can be administered. We have found that a variation in infusion rate between 3 and 10 ml per minute (0.15 to 0.5 g of glucose per minute) is generally sufficient to maintain both the sugar level and the state of consciousness at a uniform level. The adjustment of the blood sugar level by regulation of the speed of flow has proven to be surprisingly simple and reliable. Rapid bedside sugar determinations[5] together with clinical observations govern the adjustment of the rate of infusion. Arousal with the injection of 25 g of glucose by vein has been prompt and complete. The patients are then allowed to eat and drink and are carefully watched for late shock.

In these early experiments we have established the practice of rousing the patients for a minute or two, 2 or 3 times during each treatment, in order to assure ourselves of the continued reversibility of the coma. This is done merely by accelerating the infusion for a short period of time. Upon slowing the infusion once more, the subjects quickly revert to the comatose state. Under the conditions of our experiments, the transition from complete unconsciousness to the state in which the patient can answer questions, and *vice versa,* can be regulated by varying the blood sugar by as little as 2-5 mg %. We have found that the critical level for coma is generally around 40 mg %. Progressive lowering of the blood sugar below these concentrations causes deepening coma with the typical changes in level of neurological integration noted in ordinary coma.

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[4] Greeley, Paul O., *Am. J. Physiol.,* 1940, **129**, 17.

[5] Hawkins, J. A., and Van Slyke, D. D., *J. Biol. Chem.,* 1929, **81**, 459.

The method thus provides a unique opportunity for the systematic study of physiological and psychological functions at different levels of neurological integration.

These moderately prolonged comas administered at intervals of several days have resulted in improvement in patients whose psychoses were resistant to the ordinary full course of insulin treatment. The therapeutic value of the procedure must, however, await more extended study. We are gradually increasing the duration of coma in successive trials. Further modifications in the technique may become necessary. Since numerous cases of accidentally induced protracted coma have amply demonstrated its therapeutic effectiveness,[1] we hope that a single prolonged coma of one or more days, of the controlled type described above, may prove to be the equivalent of long periods of ordinary treatment. At any rate, it can be reasonably anticipated that this modification of the so-called insulin shock treatment will shorten its duration, simplify its management and still further extend its range of usefulness.

13489

Presence of Estrogenic Hormone(s) in Testicular Material.

BERT CUNNINGHAM, JOSEPHINE MAY, AND SETH GORDON.
From the Zoology Department, Duke University.

Although a number of studies have been made on male urine to determine the presence or absence of estrogens, comparatively few have been made upon testes. This is rather surprising in the light of the work of Zondek[1] who reported enormous quantities of estrogenic substance (more recently identified as alpha-estradiol and estrone by Beall[2]) in the testes of the stallion.

Zondek,[1] while more often credited with the report on the stallion testes, examined also the bull testes. Perhaps because of the relatively minute amounts (less than 0.1%) found in the latter as compared to the former it was not thought to be worth recording in the later literature. Zondek assumed that "the mass production and excretion of the estrogenic hormones in the testes is a peculiarity of the equines."

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[1] Zondek, B., *Nature*, 1934, **133**, 209.

[2] Beall, D., *Bioch. J.*, 1940, **34**, 1293.

There is considerable difference of opinion among those who have examined testes for estrogens. A number of workers have failed to secure any results from their extracts, but on the other hand sufficient data have been recorded to indicate that estrogens occur in the testes of several species. Fellner,[3] Brouha and Simonnet,[4] Dorfman, *et al.*,[5] all reported the presence of estrogens in bull testes, while Laqueur and deJongh[6] report them in human testes and Dodds, *et al.*,[7] in "mammalian" testes. With the exception of Zondek all of the workers who have found estrogens in the testes have reported only qualitative results.

The desirability of further quantitative study is evident, but any quantitative study based upon extraction and bio-assay is bound to be more or less inaccurate. About the most one can say is that there is at least as much hormone as the assay indicates.

During the course of this study we were fortunate enough to secure two lots of fresh deer testes. These were prepared and assayed in the same manner as the bull testes. We thus had 4 preparations as follows:

Extract I: 2103 g of frozen bull testes.
" II: 2119 " " " " "
" III: 671 " " fresh deer "
" IV: 531 " " " " "

The fresh or frozen material was extracted according to the Gallagher and Koch[8] method. Both androgens and estrogens were present in the crude extract and they were separated by alkali fractionation according to the Gallagher, Koch, and Dorfman[9] procedure. The alkali-estrogen portion was neutralized with HCl and again fractioned with ether. The ether was evaporated and the residue taken up in olive oil.

Ovariectomized rats were used for assay with the following results:

EXTRACT I.

1 rat given equiv. of 400 g bull testes, positive
1 " " " " 300 " " " "
4 " " " " 200 " " " all positive
2 " " " " 100 " " " both negative

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[3] Fellner, O. O. *Pflugers Arch.*, 1921, **189**, 199.

[4] Brouha, L., and Simonnet, H., *C. R. Soc. Biol.*, 1928, **99**, 41.

[5] Dorfman, R. I., Gallagher, T. F., and Koch, F. C., *Endocr.*, 1935, **19**, 33.

[6] Laquer, E., and de Jongh, S. E., *J. A. Med. Assn.*, 1928, **91**, 1169.

[7] Dodds, E. C., Greenwood, A. S., and Gallimore, E. J., *Lancet*, 1930, **228**, 683.

[8] Gallagher, T. F., and Koch, F. C., *J. Biol. Chem.*, 1929, **84**, 495.

[9] Gallagher, T. F., Koch, F. C., and Dorfman, R. I., PROC. SOC. EXP. BIOL. AND MED., 1935-36, **33**, 440.

EXTRACT II.

1 ” ” ” ” 636 g bull testes, positive
3 ” ” ” ” 424 ” ” ” all positive
1 ” ” ” ” 212 ” ” ” negative

EXTRACT III.

2 ” ” ” ” 122 g deer testes, both positive
3 ” ” ” ” 61 ” ” ” 1 neg., 2 positive
3 ” ” ” ” 30 ” ” ” all negative

EXTRACT IV.

4 ” ” ” ” 106 g deer testes, 1 neg., 3 positive

The shortage of material prevented a closer determination of the minimal effective dosage, but any correction would indicate a greater concentration of estrogens in the testes than shown herein.

It is to be noted that the second extracts of both bull and deer testes are far less potent than the first extracts. This may be due in part to the fact that in the second extracts a much larger lipoidal content was encountered. Efforts to free the hormones from the lipoid by the Thayer, Jordan and Doisy[10] procedure may not have been fully effective in our hands. We are at a loss to explain the presence of this large lipoid content since all the coverings with attached fat were removed from the testes prior to extraction.

The data confirm the observations of Fellner, Brouha and Simonnet, Zondek, and Dorfman, *et al.*, that estrogens occur in the bull testes, and the quantitative results on Extract I agree remarkably well with those of Zondek.

We have demonstrated for the first time, we believe, that estrogens occur in fresh deer testes and that they have a potency about 3 times as great per gram of tissue as those that occur in frozen bull testes.

13490

Action of Urea and Ether on Agent and Complement-fixing Antigen of *Lymphogranuloma venereum.*

Clara Nigg. (Introduced by Geoffrey Rake.)

From the Biological Laboratories of E. R. Squibb & Sons, New Brunswick, N.J.

That the complement fixation reaction is a valuable aid in the diagnosis of *Lymphogranuloma venereum* has been firmly established.[1-3] Two antigens were found to be satisfactory, viz., (a) the resuspended sediment obtained after high speed centrifugation (12,000 rpm for 2 hrs.) of suspensions of yolk sac membranes from chick embryos infected with the agent of *Lymphogranuloma venereum* and (b) suspensions of lung tissue from mice infected with the same agent. Although Rake, Shaffer, Jones and McKee[4] observed that the supernate from the yolk sac material centrifuged at high speed likewise contained significant amounts of complement-fixing antigen, the high speed sediment was nevertheless used in the earlier work because the retention of the other constituents was found to lead to anticomplementary activity. Table I shows the activity of the various fractions obtained in the course of fractional centrifugation of suspensions prepared from yolk sac membranes of infected chick embryos.[2, 5] Centrifugation even at low speed (2000 rpm for 1 hr) resulted in a considerable loss of activity from the supernate, while the activity of the low speed supernate was about equally divided between the high speed sediment and supernate respectively.

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[10] Thayer, S. A., Jordan, C. N., and Doisy, E. A., *J. Biol. Chem.*, 1928, **79**, 53.

TABLE I.
Sedimentation of the Complement-fixing Antigen of *Lymphogranuloma venereum.*

	Antigen dilutions						
Antigens	1-50	1-75	1-100	1-150	1-200	1-250	1-300
No. 17, prepared 9/8/41 tested 9/10/41							
Fraction a—Crude 10% suspension	4+	4+	4+	4+	4+	3-4+	
” c—Low speed supernate	4+	4+	4+	3-4+	3+	2+	
” d—High ” ”	4+	3-4+	3+	2+	1+	0	
” e— ” ” sediment	4+	3-4+	2+	1+	0	0	
No. 19, prepared 9/17/41 tested 9/19/41							
Fraction a—Crude 10% suspension	4+	4+	4+	4+	4+	4+	3-4+
” b—Low speed supernate	4+	4+	4+	4+	3-4+	2+	—
” c—High ” ”	4+	4+	3-4+	1+	0	0	
” d— ” ” sediment	4+	4+	3-4+	1+	0	0	

Results are given in terms of fixation.

All fractions except the high speed sediment became so anti-

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[1] McKee, Clara M., Rake, Geoffrey, and Shaffer, Morris F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 410.

[2] Rake, Geoffrey, Shaffer, Morris F., Grace, Arthur W., McKee, Clara M., and Jones, Helen P., *Am. J. Syph., Gon., and Ven. Dis.*, 1941, **25**, 687.

[3] Shaffer, Morris F., Rake, Geoffrey, Grace, Arthur W., McKee, Clara M., and Jones. Helen P., *Am. J. Syph., Gon., and Ven. Dis.*, 1941, **25**, 699.

[4] Rake, Geoffrey, Shaffer, Morris F., Jones, Helen P., and McKee, Clara M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 300.

[5] Rake, Geoffrey, McKee, Clara M., and Shaffer, Morris, F., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 332.

complementary shortly after formalin was added to inactivate the virus that all but the latter were unsuitable for use as antigens within a week or two after their preparation. The development of this anticomplementary activity is shown in Table II.

TABLE II.
Action of Formalin on Complement-fixing Activity of Yolk Sac Material.

Antigens Prepared 8/14/41	Tested	Tests with positive serum and antigens diluted:						Anticomplementary controls on antigens diluted:		
		1-50	1-75	1-100	1-150	1-200	1-250	1-50	1-100	1-200
No. 13-1 Low speed supernate of saline suspension containing formalin to 0.1%	8/18	4+	4+	4+	3-4+	3+	2-3+	0	0	0
	8/27	4+	4+	4+	4+	4+	3-4+	4+	3+	2+
No. 13-5 Low speed supernate of saline suspension containing M/45 phosphate buffer pH 7.4 and formalin to 0.1%	8/18	4+	4+	4+	4+	3-4+	3+	2+	0	0
	8/27	4+	4+	4+	4+	4+	4+	4+	4+	4+

Results are given in terms of fixation.

It seemed possible that the anticomplementary property developed as the result of the action of the formalin on nonspecific components of the yolk sac suspensions, since the property developed more rapidly and in greater amounts in the crude suspensions than in the centrifuged supernates from which a considerable amount of inactive material had been removed. The high speed sediment was never observed to become anticomplementary at icebox temperature after the addition of formalin. If, therefore, formalin was used as the inactivating agent, the only fraction suitable for complement fixation antigen appeared to be that sedimented at high speed. Since the other fractions contained considerable activity (Table I), it seemed desirable to seek other means of inactivating the virus which would not lead to the development of anticomplementary activity.

It is well known that urea is a protein denaturant and as such its action on certain viruses has been studied by a number of investigators.[6-10] Most of the reports indicate not only inactivation of the

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[6] MacKay, Eaton M., and Schroeder, Charles R., Proc. Soc. Exp. Biol. and Med., 1936-37, **35**, 74.

[7] Höring, F. O., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1939, **32**, 597.

[8] Frampton, Vernon L., and Saum, A. M., *Science*, 1939, **89**, 84.

[9] Stanley, W. M., and Lauffer, Max A., *Science*, 1939, **89**, 345.

[10] Hoyt, Anson, and Warner, Douglas, Proc. Soc. Exp. Biol. and Med., 1940, **43**, 154.

TABLE III.
Comparison of Action of Formalin and Urea on Complement-fixing Activity of Yolk Sac Suspensions.

		Tests with positive serum and antigens diluted:						Anticomplementary controls on antigens diluted:			
	Tested	1-50	1-75	1-100	1-150	1-200	1-250	1-50	1-100	1-200	1-300
Antigen No. 18											
Fraction 1											
Crude suspension (prepared 9/12/41) containing 0.1% formalin (added 9/25/41)	10/7	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Fraction 2											
Crude suspension (same as 1) containing 1% urea (added 9/12/41)	11/18	4+	4+	4+	4+	3-4+	2+	0	0		
Fraction 4											
Supernate (prepared 9/12/41 by centrifuging crude suspension 2000 rpm 1 hr) containing 0.1% formalin (added 9/25/41)	9/26	4+	4+	4+	3+	3+	1+	0	0		
Fraction 5											
Supernate (same as in 4) containing 1% urea (added 9/12/41)	9/26	4+	4+	4+	3+	2+	0	0	0		
Antigen No. 13, low speed supernate of 10% saline suspension prepared 8/14/41 and treated as follows:											
Fraction 1											
0.1% formalin added 8/14/41	8/18	4+	4+	4+	3-4+	3+	3+	0	0	0	
	8/27	4+	4+	4+	4+	4+	3-4+	4+	3+	2+	
Fraction 4											
1% urea added 8/14/41	9/4	4+	4+	4+	3-4+	3+	2-3+	0	0	0	
Fraction 6											
Kept frozen. 0.1% formalin added 9/25/41	9/26	4+	4+	4+	2-3+	1+		1+	0	0	
Fraction 7											
Kept frozen. 1% urea added 9/5/41	9/26	4+	4+	4+	3+	2+		0	0	0	

Results are given in terms of fixation.

virus but destruction so complete as to entail loss of antigenicity. In spite of these reports, the action of urea on the agent of *Lymphogranuloma venereum* in yolk sac material was investigated and it was found that as little as 1% urea inactivated the virus completely within 1-2 weeks' time at icebox temperature, and as much as 10% urea could be used without apparent loss in the complement-fixing activity of yolk sac suspensions so treated (Table III). Not only did small amounts of urea serve to inactivate the virus in the complement-fixing antigens, but even crude 10% suspensions of yolk sac material, containing all of the non-specific components failed to develop anticomplementary activity within 4 months' time at icebox temperature. The crude suspensions constituted antigens with significantly higher titers than other fractions since, as shown above, some activity was lost at each step of the differential centrifugation.

The inactivation of the virus by ether and the action of the latter on the complement-fixing antigen were similarly studied. One volume of 10% crude yolk sac suspension was shaken for ½ hr at room temperature with 2 volumes of ether. The ether supernate obtained by centrifuging at 1000 rpm for 5 min was discarded. The residual suspension was again extracted with ether in the same way. Ether was more completely removed *in vacuo*. This treatment resulted in the complete inactivation of the virus without impairment of the complement-fixing activity or the development of anticomplementary activity[11] within 4 months' time, the longest period so far tested.

Summary. 1. The agent of *Lymphogranuloma venereum* in yolk sac material can be completely inactivated by the addition of 1 or 2% urea and also by extraction with ether. 2. Crude suspensions of yolk sac material treated with urea or ether constitute antigens (devoid of anticomplementary activity) of considerably higher potency than the high speed sediments obtained from the same suspensions.

The technical assistance of Miss Augusta Hollender is gratefully acknowledged.

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[11] Howitt, Beatrice F., *J. Immunol.*, 1937, **33**, 235.

13491

Failure of Certain Growth Factors to Inhibit Sulfonamide Bacteriostasis in Simple Media.

LOWELL A. RANTZ. (Introduced by A. L. Bloomfield.)

From the Department of Medicine, Stanford University School of Medicine, San Francisco, California.

The mechanism of the action of the sulfonamide compounds in the inhibition of bacterial growth is, at the present time, imperfectly understood. Several substances, including peptones, purulent exudates,[1] methionine,[2] cozymase,[3] and para-amino benzoic acid,[4] have been shown to interfere with the bacteriostatic activity of these chemicals. It is now believed that para-amino benzoic acid is an essential growth factor which is required by most bacteria and synthesized by them.[5] It has been isolated from certain microörganisms. Because it resembles the sulfonamides chemically, and is an exceedingly potent inhibitor of their bacteriostatic activity, it is possible that the primary action of these chemicals on bacterial growth lies in their ability to interfere with the function of para-amino benzoic acid in the metabolism of these organisms.

Many other chemicals are also known to be essential to bacterial metabolism and several have been studied for possible activity as inhibitors of sulfonamide bacteriostasis. The most recent report is that of Strauss, Dingle and Finland,[6] who studied nicotinamide, thiamin, biotin, and yeast nucleic acids, and showed that these substances were without activity as sulfonamide inhibitors when tested in simple media.

It is the purpose of this report to describe somewhat similar observations which were begun before the appearance of their report, the growth factors studied being thiamin chloride, nicotinic acid, cocarboxylase, pyridoxine, riboflavin, calcium pantothenate and para-amino benzoic acid.*

Methods. Two organisms, *E. coli* and *Staphylococcus aureus* were used in these experiments. Casein hydrolysate known to be free of sulfonamide inhibitors was used as a nitrogenous base for the simple media used in the tests. Staphylococci grew well in a modification of the media described by Knight.[11] The observations on *E. coli* were conducted in a medium which contained .5% of ammonium sulphate, .5% of sodium chloride, .2% of dextrose, .3% of potassium diphosphate, and .2% of hydrolyzed casein in distilled water. Both media were adjusted to pH 7.6. The growth factors were dissolved in distilled water adjusted to pH 7, sterilized by Seitz filtration and added in serial dilutions to the base medium. A heavy growth of organisms within 24 hours was always obtained. Sulfathiazole was added to the medium in a concentration of 2.5 μg per milliliter, which produced marked bacteriostasis, but permitted slight visible growth within 48 to 72 hours. In each experiment a constant number of organisms were added to every culture tube, usually about 50 per milliliter.

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[1] MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

[2] Bliss, E. A., and Long, P. H., *Bul. J. H. Hosp.*, 1941, **69**, 14.

[3] West, R., and Coburn, A. F., *J. Exp. Med.*, 1940, **72**, 91.

[4] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

[5] Fildes, P., *Lancet*, 1940, **1**, 955.

[6] Strauss, Elias, Dingle, J., and Finland, M., *J. Immunol.*, 1941, **42**, 313, 331.

* Obtained through the courtesy of Merck and Company, Rahway, New Jersey.

The rate of bacterial growth under control and test conditions was determined by measuring the time of appearance of visible growth and the density of the cloud of organisms at intervals by means of a photoelectric nephelometer. The values obtained in this manner have been shown to be proportional to the number of viable organisms present during the period of rapid bacterial growth.

Results. Nicotinic Acid. Nicotinic acid has been shown to be an essential growth factor for many bacterial species, including *Staphylococcus aureus, C. diphtheriae, Shigella paradysenteriae,*[7] and *Brucella abortus.*[8]

The basal media described above failed to support the growth of *S. aureus* in the presence of an excess of thiamin chloride in the absence of nicotinic acid. The addition of 1 microgram per cubic milliliter was sufficient to cause the development of maximal bacterial growth, no further increase being induced by concentrations of the chemical to 100 μg per milliliter; 1000 μg per milliliter strikingly interfered with the rate of multiplication, but failed to prevent the eventual development of maximal growth. No effect of sulfonamide bacteriostasis was demonstrated in media containing from 1 to 100 μg per milliliter of nicotinic acid. The base media supported luxuriant growth of *E. coli* in the absence of additional growth factors. Nicotinic acid in concentrations of 100 μg per milliliter failed to enhance the rate of growth of control preparations and had no effect whatsoever on sulfonamide bacteriostasis.

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[7] Koser, S. A., and Saunders, F., *Bact. Rev.*, 1938, **2**, 99.

[8] Koser, S. A., Breslove, B. B., and Dorfman, A., *J. Infect. Dis.*, 1941, **69**, 114.

[11] Knight, B. C. J. G., *Brit. J. Exp. Path.*, 1941, **16**, 315.

Thiamin Chloride. Thiamin has been shown to be essential to the growth of *S. aureus,* certain molds,[7] and *Brucella abortus,*[8] among other bacteria. The base medium used in these experiments in the presence of excess nicotinic acid supported minimal growth of *S. aureus* and was, therefore, not entirely free of thiamin. The addition of .00001 μg per milliliter of this chemical as the chloride was necessary to obtain optimal growth. A further increase in concentration to 100 μg per milliliter failed to further increase, and concentration greater than 1000 μg decreased, the rate of growth. Within the range of optimal growth, increasing amounts of thiamin chloride in the medium had no effect on sulfonamide bacteriostasis.

Neither the growth rate of *E. coli* or sulfonamide bacteriostasis of this organism was affected by the presence, in the base medium, of 100 μg per milliliter of thiamin chloride.

Cocarboxylase. Diphosphothiamin (Cocarboxylase) is an essential intermediary substance formed during the metabolism of thiamin. It was substituted for thiamin chloride in the same concentrations in a series of experiments similar to those just described. The results with both *S. aureus* and *E. coli* were approximately the same in every respect as those obtained with thiamin chloride.

Riboflavin. Riboflavin has been shown to be essential to the development of certain lactic acid bacteria[7] and streptococci.[9] Its addition to the basal media containing excess thiamin chloride and nicotinic acid in concentrations up to 200 μg per milliliter failed to enhance the rate of bacterial growth of *S. aureus* or to interfere with sulfonamide bacteriostasis. Similar results were obtained with *E. coli* growing in the base medium to which no additional growth factors other than riboflavin had been added.

Pyridoxine. Pyridoxine (Vitamin B6) appears to be a growth factor for certain streptococci[9] and other organisms. In experiments similar to those conducted with riboflavin, its addition to the base media in concentrations up to 1000 μg per milliliter failed to enhance the rate of bacterial growth or interfere with sulfonamide bacteriostasis.

Pantothenic Acid. Pantothenic acid has been shown to be essential to the growth of certain lactic acid bacilli, yeasts, and hemolytic streptococci,[9] as well as other microörganisms. The addition of this substance as the calcium salt to the simple medium containing excess thiamin and nicotinic acid used in these experiments slightly but definitely increased the rate of multiplication of *S. aureus* over that obtained in its absence. A concentration of 200 μg per milliliter was necessary to obtain this effect. Attempts were made to demonstrate a similar augmentation of the rate of growth of *E. coli* but were unsuccessful.

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[9] Woolley, D. W., *J. Bact.*, 1941, **42**, 155.

Sulfonamide bacteriostasis of *S. aureus* and *E. coli* was not affected by concentrations of calcium pantothenate up to 200 μg per milliliter.

Para-amino Benzoic Acid. Para-amino benzoic acid is known to be a powerful inhibitor of sulfonamide bacteriostasis, a constituent of the cells of certain yeasts,[4] and an essential growth factor for at least one of the Clostridia.[10]

In these experiments the addition of this chemical to the basal media in concentrations up to 100 μg per milliliter failed to enhance the growth of either *S. aureus* or *E. coli.* As was to be expected, para-amino benzoic acid markedly inhibited sulfonamide bacteriostasis, .001 μg per milliliter being adequate for the inhibition of 2.5 μg per milliliter of sulfathiazole.

Summary and Conclusions. Nicotinic acid, thiamin chloride, cocarboxylase, riboflavin, pyridoxine and para-amino benzoic acid have been shown to have no effect on the photometrically determined growth rate of *S. aureus* and *E. coli* in simple media. Calcium pantothenate definitely accelerated the rate of multiplication of *S. aureus* but not of *E. coli* when present in a concentration of 200 μg per milliliter. None of these substances except para-amino benzoic acid antagonized sulfathiazole bacteriostasis although present in amounts 40 times as great as the sulfonamide. Concentrations of sulfonamide 2,500 times that of the accompanying para-amino benzoic acid were completely ineffective in inhibiting bacterial growth.

These observations, therefore, support the view that the sulfonamides directly affect that part of the microbial metabolic system which specifically utilizes para-amino benzoic acid, since relatively large amounts of 6 other important growth factors completely failed to inhibit their bacteriostatic activity.

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[10] Rubbo, S. D., and Gillespie, J. M., *Nature,* London, 1940, **146**, 838.

13492

Comments on a Newly Recognized Group of the Meningococcus.

SARA E. BRANHAM AND SADIE A. CARLIN.

From the Division of Biologics Control, National Institute of Health, Bethesda, Maryland.

For practical purposes, meningococci may be considered to fall into two broad groups, I and II. Group I, which includes the Types I and III of Gordon and Murray, has apparently been responsible for all of the epidemics of which there is serological knowledge for the past 20 years,[1] whereas Group II strains have been most common in sporadic cases and in chronic carriers.

The last peak in the number of cases of meningococcus meningitis was in 1936, and 90% of the strains sent to the National Institute of Health during that year for typing were found to belong to Group I, and only 9% to Group II. In 1937 the epidemic wave began to subside, and the number of Group I cases rapidly decreased. Group II strains increased to 16% during 1937, to 46% in 1938, and to 55% in 1939. During this time conspicuous differences among so-called Group II strains were noted. Pittman noted that there were no antibodies for certain strains in many polyvalent antimeningococcus sera sent routinely to the Institute.

For several years it has been obvious that most polyvalent antimeningococcus sera have given little protection against Group II infections.[2] It seems that either members of this group were poor antigens, the wrong animals and methods were used, or else the strains comprising the group differed among themselves more than had been known.

During the 1937-40 period when most of the cases of meningococcus meningitis were sporadic, the absence of protective antibodies for Group II became even more evident. It seemed essential to find really antigenic strains.

With this end in view, monovalent sera were prepared by immunizing rabbits, in duplicate, with 12 newly isolated Group II strains. Virulence was maintained by occasional passage through mice. For immunization, carefully graduated doses of 5-hour blood agar slant cultures were given intravenously on 3 successive days of each week, with a week's rest period and a trial bleeding at the end of

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[1] Branham, S. E., and Carlin, Sadie A., *J. Bact.*, 1937, **34**, 275.

[2] Branham, S. E., Proc. 29th Annual Meeting of the Drug Mfg. Assn., 1940, p. 210.

6 weeks. The first 3 injections contained approximately 10,000,000 meningococci each. Sera from the trial bleedings were studied by agglutination at 37°C, by "halo" production,[3] and by mouse protection.[4]

In rabbits immunized with 4 of the strains, protective antibodies could be demonstrated within a few weeks. In the others, practically none could be found, even for the homologous strains, after a year and a half of immunization.

The 4 strains that seemed to be good antigens were studied further. Sera prepared with them protected mice against homologous strains to a high degree—*i. e.,* 0.001 cc protected against 100,000 minimum fatal doses. Moreover, they protected equally well against each other but not against any other strains that were included in the first experiments.

It was obvious that these 4 strains were immunologically alike though they came from widely separated localities, and that they were different from the other Group II strains. From this time on all new meningococci that seemed to fall into Group II by agglutination were studied with these sera by means of the mouse protection test.

Twenty-four of 43 Group II strains (53%) received since that time have been found to be immunologically identical with these 4. Protection tests have been definite and clear-cut. No other sera have protected against them and no strains other than these 24 have been responsive to the sera from 4 original strains. A summary showing the sources is given in Table I.

Although these meningococci may be agglutinated to some extent by any broad Group II serum, it is easy to prepare specific agglutinating serum if smooth strains and brief immunization periods are used. It has been possible to classify them by specific agglutination when they are newly isolated using 37°C incubation for 2 hours, followed by ice-box storage overnight. This "typing" is usually confirmed by mouse protection. Plate precipitation or "halo" production is distinctive also when horse serum is used, but immune rabbit sera have a tendency to show halos with all Group II strains.

The heterogenicity of Group II meningococci has been noted by others. A positive "Quellung" reaction has been observed[5] by some strains with appropriate sera whereas the regular Group II strains

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[3] Pittman, Margaret, Branham, Sara E., and Sockrider, Elsie M., *U. S. Public Health Rep.*, 1938, **53**, 1400.

[4] Branham, Sara E., and Pittman, Margaret, *U. S. Public Health Rep.*, 1940, **55**, 2340.

[5] Cohen, Sophia M., *J. Inf. Dis.*, 1940, **67**, 74.

TABLE I.
Source and Distribution of II Alpha Strains of Meningococci.

No.	Laboratory designation	Date received		
1	1054	6/3/37	Spinal fluid	Baltimore, Md.
2	1166	3/4/40	"	N. Y. State Lab.
3	1170	3/21	"	Syracuse, N. Y.
4	1171	3/28	"	Washington, D. C.
5	1184	12/27	"	Baltimore, Md.
6	1189	2/1/41	"	Nashville, Tenn.
7	1191	2/3	"	Seattle, Wash.
8	1194	2/14	"	Syracuse, N. Y.
10	1195	2/15	"	Galveston, Texas
11	1207	2/27	"	Nashville, Tenn.
12	1218	3/7	Nasopharynx	Camp Devens, Mass.
13	1219	3/7	"	"
14	1220	3/7	"	"
15	1223	3/7	"	Halifax, N. S.
16	1225	3/7	"	"
17	1252	5/6	Spinal fluid	Minneapolis, Minn.
18	1259	6/5	"	Washington, D. C.
19	1271	6/12	Nasopharynx	Ft. Geo. Meade, Md.
20	1279	6/30	Spinal fluid	Minneapolis, Minn.
21	1284	7/15	"	Cheyenne, Wyo.
22	1286	7/14	Blood	Syracuse, N. Y.
23	1294	10/8	Nasopharynx	Boston, Mass.
24	1296	10/27	Sputum	Baltimore, Md.

have not been found to show this phenomenon. Cohen[5] placed the capsulated strains tentatively in her "Group X" of atypical meningococci. In the 1940 report of the New York State Board of Health, Cohen[6] reported that about one-fourth (25%) of the Group II meningococci from New York State have fallen into the encapsulated group.

Undoubtedly, a distinct immunological entity is involved. It is related to Group II by agglutination but is quite distinct on a basis of protection. There is sometimes a very slight agglutinin relation to Group I.

It has been impossible to demonstrate a relationship between these organisms and the Type IV of Gordon and Murray[7] though their original type strain and other more recent strains have been studied. Rake's[8] Type VII, reported as being related to Group II, has been unavailable for comparison.

Since the meningococci just described have comprised one-fourth (25%) of the II's found in New York State, and one-half (53%) of the II's received by the National Institute of Health from the country at large, they apparently form a group of epidemiological and clinical importance. Since they are a homogeneous group immunologically, and are excellent antigens, the most obvious explanation for the failure of polyvalent therapeutic sera to protect against such infections is that these strains have not been recognized as being different and have not been included in immunization of the animals which provided the serum.

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[6] Cohen, Sophia M., *Ann. Rep. N. Y. State Div. Lab. and Res.*, 1940, p. 27.

[7] Gordon, M. H., and Murray, E. G. D., *J. Roy. Army Med. Corps*, 1915, **25**, 411.

[8] Rake, Geoffrey, *J. Exp. Med.*, 1934, **59**, 553.

In order that such strains and their significance may be recognized, it would be helpful to employ a convenient designation. The term "encapsulated II's" is awkward and also misleading, since there are often strains of this group in which capsules are not demonstrable. The term "Group X" is not sufficiently definite and does not indicate its relationship.

The first person to report immunological differences among the Group II meningococci was Dopter[9] when he described *alpha, beta* and *gamma* types of his "parameningococcus", naming them in the order of their frequency. Unfortunately, Dopter's original strains are no longer in existence but his idea offers a good precedent to follow. Since the strains just described comprise what is apparently the largest homogeneous unit among those related to Group II, they have been designated in this laboratory as Group II *alpha,* considering them tentatively as a sub-group of II. This apparent serological relation to Group II may be very superficial. Crossing in agglutination and in halo production with some rabbit sera are the only evidence for kinship.

Whether these are really a sub-group of II or something quite independent, only time and further study can tell. It will be easy to make a final classification when enough is known. Since Dopter's designation was the earliest and is applicable, it can well be brought into use for the present time.

Summary. About 50% of the strains of meningococci which have been superficially "typed" as belonging to Group II have been found to form an independent and homogeneous lot. Those who have worked with individual strains agree that many of them give a definite Quellung reaction, they are good antigens, and produce sera that protect mice well against infections by meningococci of the same group. But there is no cross protection with sera representing other groups.

Because of the close agglutinogenic relation to other Group II meningococci, it is suggested that, for the present, they be designated as Group II *alpha,* following the precedent set by Dopter in his studies of his parameningococcus since designated as Group II.

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[9] Dopter, Ch., and Pauron, *C. R. Soc. de Biol.*, 1914, **77**, 231.

13493

Thiamin Requirement of Albino Mice.

JAMES D. HAUSCHILDT. (Introduced by H. Jensen.)

*From the Department of Physiological Chemistry, University of Washington, Seattle, Wash.**

Since the preparation of thiamin in pure form, no investigations of the thiamin requirement of the albino mouse have come to the attention of the writer. Previously, Beard[1] and Bing and Mendel[2] had determined that albino mice required yeast or a suitable extract of yeast or rice polishings for normal growth and prevention of polyneuritis, indicating that mice were susceptible to a deficiency of Vitamin B_1. Likewise Freudenberg and Cerecedo[3] have recommended the white mouse as a test animal for antineuritic concentrates and recently Foy and Cerecedo[4] have determined the growth response of the mouse to various combinations of crystalline B vitamins. In the course of other work at this laboratory it was found that the addition of thiamin to a diet adequate in the fat-soluble vitamins and minerals and containing autoclaved yeast as a source of water-soluble factors made the diet capable of sustaining normal growth in young mice. As a result of this finding it was considered possible to determine the minimal thiamin requirement of the albino mouse using normal growth as a criterion of adequacy.

Experimental. The mice used in these experiments were from a local albino strain used by other departments at this university for various types of investigation, and the strain had been inbred for several years at the time of writing. The normal growth rate was obtained by weaning the mice at 21 days of age on a commercial diet supplemented with occasional fresh meat and lettuce. The basal diet used in the rest of the experiments upon thiamin requirement consisted of sucrose 65, casein 18, butterfat 2, cod liver oil 1, Osborne-Mendel salts 4, and autoclaved yeast 10. The casein was extracted continuously for 96 hours with hot 96% alcohol, then twice with cold 60% alcohol for 16 hours each time. Dry powdered "Yeast Foam" was autoclaved for 5 hours at 15 lb pressure.

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* Present address, Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

[1] Beard, H. H., *Am. J. Physiol.*, 1926, **75**, 668.

[2] Bing, F. C., and Mendel, L. B., *J. Nutrition*, 1929, **2**, 49.

[3] Freudenberg, W., and Cerecedo, L. R., *J. Biol. Chem.*, 1931, **94**, 207.

[4] Foy, J. R., and Cerecedo, L. R., *J. Nutrition*, 1941, **22**, 439.

Mice weighing 9 to 10 g at 21 days of age were weaned on the basal diet. Crystalline thiamin was added to the basal diet after a depletion period of 9 to 11 days. The group at each level contained approximately an equal number of males and females. Rats, used as an additional control, were weaned on the basal diet when weighing 42-48 g and after a depletion period of 19 days were given crystalline thiamin as a supplement. Both rats and mice were weighed at 5-day intervals.

Results. Growth responses of mice are shown in Fig. 1. Weight changes are shown beginning at the end of the depletion period. When administration of crystalline thiamin was begun the weight

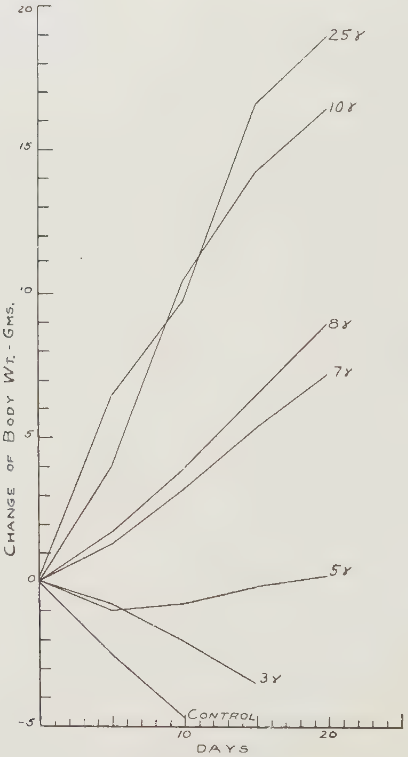

FIG. 1.

Weight change of albino mice receiving various amounts of thiamin. Curves represent average gain or loss of all animals in each group. Groups are as follows:

µg	Animals
3	11
5	9
7	11
8	11
10	3
25	3
0	4

of all animals had remained constant or had begun to decline.

Thiamin added to the basal diet is shown to produce growth in accordance with the amounts administered. Five micrograms per day per animal is sufficient for maintenance of body weight but insufficient to allow growth over the test period. Subnormal weight increases take place when thiamin is given at levels of 5 to 10 μg per animal per day. Ten micrograms daily produced normal growth. All animals receiving thiamin maintained a healthy appearance during the experimental period. No distinction is made between sexes because growth rates of both sexes are the same over the range indicated in this experiment.

Rat controls grew normally when receiving 10 μg of thiamin per day each. Basal diet controls lost weight and showed characteristic symptoms of acute thiamin deficiency after 15-20 days on the basal diet. The diet was thus shown to be deficient in thiamin as judged by the response of both rats and mice receiving it.

Summary. The amount of thiamin required for growth of albino mice has been determined. Five micrograms per mouse per day was found to maintain body weight attained at the time of weaning. Ten micrograms per mouse per day is the minimal level for normal growth. Less than 5 μg per mouse per day results in loss of weight and eventual death.

13494

Toxicity of Potassium and Phosphate for Normal and Adrenalectomized Rats.

S. Rapoport and George M. Guest. (Introduced by S. Tashiro.)

From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati, O.

Several investigators[1] have shown that the potassium ion has a greater toxicity for adrenalectomized animals than for normal animals, and have suggested that this increase in toxic effects of potassium might be due to the derangement of normal relationships between extra- and intracellular electrolytes that follows adrenalectomy. In such a condition one might also expect the phosphate ion to have an increased toxicity since phosphate is an important intracellular anion with metabolism closely related in some respects to that of potassium. Such considerations stimulated the investigations here reported.

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[1] Fenn, W. O., *Physiol. Rev.*, 1940, **20**, 377.

Experimental. Male rats from the stock of Sprague and Dawley, weighing 170 to 210 g, were used. They were fed Bills' modification of Steenbock's stock diet[2] a week and then both adrenals were removed under ether anesthesia. Food was withdrawn the third day after operation, and 24 hours later solutions containing potassium and/or phosphate were injected intraperitoneally. The toxicity of these solutions was judged by the amounts of potassium and/or phosphate which killed the animals in less than 24 hours. Three solutions were employed, containing respectively 0.5 molar concentrations of: A, potassium chloride; B, a mixture of one part KH_2PO_4 and 4 parts Na_2HPO_4, of pH 7.4; C, a mixture of one part NaH_2PO_4 and 4 parts Na_2HPO_4. Two dosages of each solution were employed: 1, an amount deemed just sufficient to kill all experimental animals, as determined in preliminary experiments; and 2, about three-fourths of this dose. The animals were left without food 24 hours after the injection. If they survived this period, feeding was resumed. Practically all animals that survived the injections 24 hours, the time limit of the test, lived 3 to 5 days longer. Normal control animals were injected in the same manner, after a 24-hour fast.

Results. The data listed in Table I indicate that on a molar basis the toxicity of the phosphate ion is approximately equal to that of potassium for normal and adrenalectomized rats. Differences in rates of absorption might explain the somewhat longer survival time of rats injected with phosphate, compared with the survival of rats injected with KCl. The lethal dose of both phosphate and potassium for adrenalectomized rats was about one-fourth their lethal dose for normal rats. The results found following injections of solution B, containing both potassium and phosphate, suggest accumulative effects.

Sodium chloride was similarly injected into adrenalectomized rats in amounts up to 10 mM per kilo, and double that amount into normal rats. None of these died, whereas 2.6 mM of sodium phosphate per kilo regularly killed adrenalectomized rats.

The effect of the preliminary administration of cortical extracts and of NaCl solution on the toxicity of phosphate in adrenalectomized rats also was studied in a few experiments. Seven adrenalectomized rats, following operation, were injected twice daily with 1 cc doses of cortical extract (Upjohn) up to the day of injection with

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[2] Bills, C. E., Honeywell, E. M., and MacNair, W. A., *J. Biol. Chem.*, 1928, **76**, 251.

TABLE I.
Lethal Effects of Intraperitoneal Injections of Potassium and Phosphate in Normal and Adrenalectomized Rats.

					Died within 24 hrs		
Rats			Amts injected			Survival time	
Preparation	No.	Sol.*	Potassium mM/kg	Phosphate mM/kg	No.	Range, hr	Avg, hr
Normal	10	A	10	0	9	0.5- 4	1.9
	10	A	9	0	5	1 - 3	1.6
	10	B	2	10.3	8	3 -15	9.4
	10	B	1.75	9	3	6.5-23	17.1
	10	C	0	11	9	1.5-12	4.8
	10	C	0	9	2	3 - 5	4.0
Adrenalectomy	10	A	3	0	10	0.8-14	3.3
	10	A	2.5	0	7	1 -23	5.5
	10	B	0.5	2.6	10	2.5-10	5.4
	10	B	0.38	1.9	8	4.5-22	9.3
	10	C	0	2.6	10	3.5-23	14.0
	10	C	0	1.9	6	4.5-22	16.7

*Test solutions: 0.5 molar concentrations of A, KCl; B, 1 part KH_2PO_4 + 4 parts Na_2HPO_4; C, 1 part NaH_2PO_4 + 4 parts Na_2HPO_4.

the phosphate solutions. Three rats received an amount of solution B containing 0.75 mM of K and 4 mM of P, and 4 rats received a dose containing 1 mM of K and 5.3 mM of P, all injected intraperitoneally. All animals survived the injections 5 to 7 days. Another group of 5 animals were given 1% NaCl solution as drinking fluid following adrenalectomy; when these were injected with a dose of solution B containing 0.6 mM of K and 3.3 mM of P, all survived for periods exceeding 24 hours.

The results suggest that the derangement of normal relationships between extra- and intracellular electrolytes that follows adrenalectomy involves both phosphate and potassium, the principal intracellular ions.

Summary. In adrenalectomized rats the toxicity of phosphate injected intraperitoneally is increased, and approximates that of potassium.

13495

Effect of Thyrotropic Hormone on Blood Cholesterol of Thyroidectomized Rabbits.

KENNETH B. TURNER AND ARLENE DELAMATER.

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.

There is general agreement that the administration of thyroid substance produces a lowering of the cholesterol level in the blood of man and experimental animals. As might be expected, this drop occurs in the absence of the thyroid gland in man[1] and in rabbits.[2]

The thyrotropic hormone of the anterior pituitary is also capable of causing a fall in the blood cholesterol.[3] The purpose of the present investigation was to determine whether this action of the hormone persisted in rabbits after the complete removal of the thyroid glands.

Dutch belted rabbits from 4 to 6 months old were fed 1 g of crystalline cholesterol thrice weekly mixed with moistened grain for an average of 8 months previous to and throughout the experimental period. The whole blood cholesterol of each animal was determined by the method of Bloor, Pelkan, and Allen[4] weekly during the preliminary period and every 2 days after the experiment began.

Five rabbits were used as controls. Eleven were operated upon and the thyroids removed under ether anesthesia from 1 to 4 months before injections were started. At the end of the experiment, an autopsy was performed. Regenerated thyroid tissue is ordinarily obvious on inspection because of its brownish red color that is sharply in contrast to the red of the muscles and other structures. In case of doubt a section was made for microscopic examination. It was found that the thyroids had been completely removed in 6 animals, but in 5 the thyroidectomy had been incomplete and more or less regeneration of thyroid tissue had taken place. These have been designated as "partially thyroidectomized".

The thyrotropic hormone was obtained through the courtesy of the Schering Corporation. It was injected subcutaneously daily for 10 to 14 days in doses of 9 mg per day except for 4 rabbits (Nos. 368, 390, 393, 394) that were given 3 mg doses.

Each rabbit was weighed before and after the series of injections.

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[1] Blumgart, H. L., and Davis, D., *Endocrinology*, 1934, **18**, 693.

[2] Turner, K. B., Present, C. H., and Bidwell, E. H., *J. Exp. Med.*, 1938, **67**, 111.

[3] Fenz, E., and Zell, F., *Klim. Wchnschr.*, 1936, **15**, 1133.

[4] Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, 1922, **52**, 191.

TABLE I.
Effect of Thyrotropic Hormone on Blood Cholesterol.

		Weight		Blood Cholesterol															
Rabbit	Sex	Before hormone kg	After hormone kg	Avg during control mo. (mg/100 cc)	Days of hormone injection (mg/100 cc) 2	4	6	8	10	12	14	Max. decrease, %	Avg during hormone inj., (mg/100 cc)	Days after hormone (mg/100 cc) 2	4	6	8	10	12	14
Intact																				
368*	M	1.5	1.1	591	467	521	527	549	—			21	516	503	(died)					
390*	M	2.0	1.9	491	515	410	481	471	—			14	469	444	—	358	—	—	—	409
378	F	2.1	1.7	477	319	346	356	376	—			33	349	366	(died)					
388	F	1.8	1.2	595	—	361	398	391	—			39	383	409	621	519	(died)			
411	M	2.4	2.4	656	525	410	456	421	252	—		62	413	268	268	207	228	164	156	124
Avg				562								45	426							
Partially thyroidectomized																				
393*	M	1.9	1.7	607	527	487	417	404	—			33	459	433	—	485	—	—	—	—
387	F	2.3	2.2	683	446	513	467	322	—			53	437	336	700	597	700	—	630	—
400	M	1.8	1.6	603	521	565	600	625	670	—	450	25	572	625	575	381	—	520	550	430
415	M	2.1	1.9	456	306	312	262	236	200	—	250	56	261	237	248	316	—	344	382	408
424	M	1.6	1.5	345	535	286	241	262	211	139	—	60	279	76	135	238	253	204	174	132
Avg				539								49	402							
Thyroidectomized																				
394*	M	1.8	1.8	341	431	312	313	278	—			18	334	325	—	309	—	—	—	371
336	F	2.0	1.9	470	352	476	505	446				25	445	433	424	515	726	—	519	—
408	F	1.6	1.5	460	506	450	348	284	475			38	413	220	214	230	213	230	214	338
412	M	1.8	1.6	502	515	284	338	340	310	318	—	43	351	261	310	420	—	500	—	565
452	F	1.5	1.6	406	375	342	228	264	311	—		44	304	315	350	421	—	357	—	—
457	M	1.8	1.7	450	371	368	226	272	211	—		53	290	318	375	347	—	383		—
Avg				438								41	356							

*Received 3 mg doses of hormone

The results are given in Table I. The average level of blood cholesterol during the month preceding the administration of the hormone is indicated for each rabbit. A hypercholesterolemia due to the cholesterol feeding was present in each instance. It is apparent that the injection of thyrotropic hormone produces a fall in the level of cholesterol in the blood regardless of the presence or absence of thyroid tissue. The effect is less marked when 3 mg of the hormone is given than with 9 mg per day.

The drop in blood cholesterol may be summarized:

	Avg during Control month, mg	Avg during hormone inj., mg	Decrease in avg, %	Max. decrease, %
Intact rabbits	562	426	24	14-62
Partially thyroidectomized	539	402	25	25-60
Thyroidectomized	438	356	19	18-53

Following the period of hormone injection the blood cholesterol tended to rise. In rabbit No. 411 of the control group, however, the cholesterol fell progressively after the injections were stopped. At autopsy the thyroids were 3-4 times normal size and extremely vascular. Unfortunately no sections were made.

The mechanism by which the thyrotropic hormone lowers the blood cholesterol in the absence of the thyroid gland remains to be elucidated. It has recently been shown[5] that the increase in oxygen consumption caused by the thyrotropic hormone also persists after thyroidectomy.

It is of interest that the hormone had little or no effect on the weight of the rabbits whose thyroids had been completely or partly removed. In 3 of the 5 intact animals a substantial weight loss occurred and was followed by death 2 to 7 days after the hormone was discontinued.

Summary. Thyrotropic hormone produces a fall in the blood cholesterol of hypercholesterolemic rabbits regardless of the presence or absence of the thyroid glands.

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[5] Billingsley, L. W., O'Donovan, D. K., and Collip, J. B., *Endocrinology*, 1939, **24**, 63.

13496

Persistence of Vaccinia Virus and Chick-Embryonic Cells in Suspended Cell Tissue Cultures.

John F. Enders and Alfred L. Florman.*

From the Department of Bacteriology and Immunology, Harvard Medical School and School of Public Health, Boston, Mass.

Introduction. It has recently been shown that in roller tube cultures various tissues would actively multiply and support the growth of viruses for unexpectedly long periods of time.[1, 2, 3] For purposes of comparison, it became of interest, therefore, to determine more exactly than had been done hitherto the period of survival of both virus and tissue cells in suspended cell cultures (Maitland,[4] Rivers,[5] Zinsser and Schoenbach[6]) which for several years have been routinely used by many investigators. As pointed out by Feller, Enders and Weller,[1] there is no evidence in the literature for the survival of vaccinia virus in such cultures beyond the 12th day.

Method. Seventy cultures were set up essentially according to the technic described by Rivers.[5] One-tenth gm of minced 10-day chick embryo was placed in a 50 cc Erlenmeyer flask containing 5 cc of Tyrode's solution and 0.005% phenol red. To 35 such flasks 0.25 cc amounts of a freshly prepared emulsion in infusion broth of vaccinia-infected chick chorio-allantoic membrane were added. The virus was a dermal strain originally obtained from the Massachusetts Antitoxin Laboratory.[7] Part of the inoculum was stored at –17°C for subsequent titration. All of the flasks were closed tightly with rubber stoppers and placed in the incubator at 37°C. At regular intervals thereafter some of the tissue fragments were withdrawn and 10 plasma clot transplants were made from each to test for viability of cells. These transplants were made by transferring ten small bits of tissue to a Petri dish, removing the excess fluid and then covering each with a drop of chicken plasma and a drop of embryonic extract. The plates were sealed with a mixture of equal parts of paraffin and petroleum jelly. All plates were incubated at 37°C and observed for 4-5 days. The appearance of any typical newly developed fibroblasts at the margin of the fragment was taken as a criterion for the presence of viable cells. The remaining material in each of the flasks inoculated with virus was stored at –17°C and, after several had accumulated, a titration of the contents on the skin of the rabbit was carried out in the usual manner.

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* Fellow of the Dazian Foundation for Medical Research.

[1] Feller, A. E., Enders, J. F., and Weller, T. H., *J. Exp. Med.*, 1940, **72**, 367.

[2] Gey, G. O., and Bang, F. B., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 393.

[3] Pearson, H. E., and Enders, J. F., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 140.

[4] Maitland, H. B., Laing, A. W., and Lyth, R., *Brit. J. Exp. Path.*, 1932, **13**, 90.

[5] Rivers, T., *J. Exp. Med.*, 1933, **58**, 635.

[6] Zinsser, H., and Schoenbach, E. B., *J. Exp. Med.*, 1937, **66**, 207.

[7] Robinson, E., *Virus and Rickettsial Diseases,* Harvard School of Public Health Symposium Volume, Harvard University Press, Cambridge, Mass., 1940.

Results. As indicated on the chart, cells from both inoculated and uninoculated cultures were viable for as long as 30 days but not after this time. After 20 days there was uniformly more evidence of viability in the control cultures.

Virus was detected in the material removed from all the infected cultures up to 20 days and from one even after 27 days, but not thereafter.

Discussion. That at least some cells survived for over 4 weeks in a medium consisting of Tyrode's solution was somewhat surprising because of the findings of Maitland, Laing and Lyth[4] and Zinsser and Schoenbach,[6] who were unable to demonstrate in this type of medium cellular respiration by the Warburg technic after 3 to 6 days. Possibly this discrepancy may be due to the incapacity of the respirometer to register the respiration of the very few viable

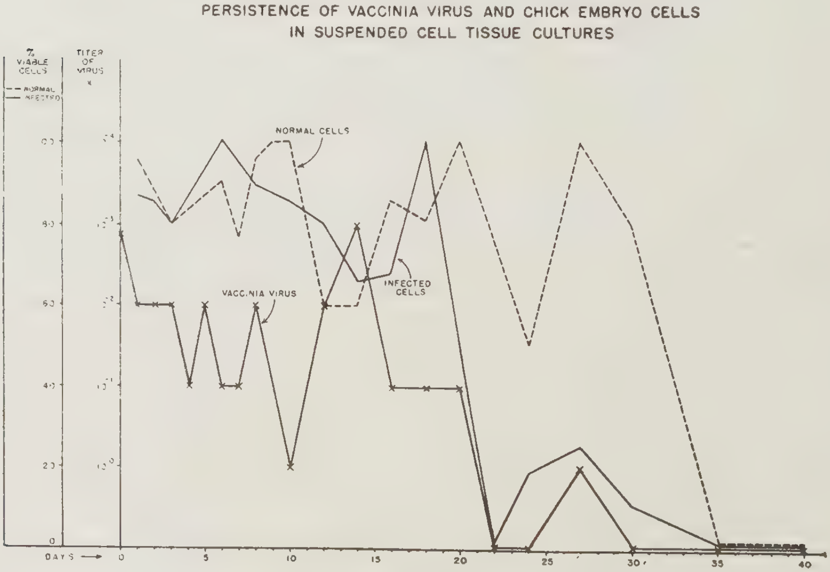

Fig. 1.

cells which may remain after incubation has been continued for several days, although Zinsser and Schoenbach failed to cultivate tissue fragments removed from the flask at a time when respiration had apparently ceased. However this may be, our experimental results left no doubt that living cells remained in the cultures for about 30 days. Not only did direct cultivation of the tissue fragments in plasma clots indicate this, but the fact that in cultures containing vaccinia virus, death of the tissue and disappearance of the virus occurred at approximately the same time.

The percentage of fragments of viable tissue removed from cultures containing virus was definitely less after the 22nd day of cultivation in comparison to uninoculated cultures. This fact suggests either that the virus may exert a more injurious effect on the cells as their vital processes become impaired or it may be possibly accounted for by differences due to random sampling. In roller tube cultures[1] where more suitable conditions for cell growth and metabolism are provided, no definite evidence was obtained of injury to the cells by the virus of vaccinia even after contact for 9 weeks.

Although in the suspended tissue medium the cells and virus both remained active longer than was expected on the basis of observations by other workers, in contrast to the roller tube method, increase of the virus was only moderate in degree and ceased after a relatively short time with resultant disappearance of viral activity.

In regard to the curve presented in this paper, it is of interest to note that in another experiment not recorded here but similar in all respects except that about 1 g of tissue was employed instead of about 0.1 g neither virus nor living cells could be demonstrated after 15 days. Once more this emphasizes the importance of adjusting the quantity of tissue in cultures of suspended tissue fragments and may account for the fact that Plotz and Ephrussi[8] were unable to observe viable cells in similar cultures after 12 to 13 days.

Conclusions. At 37°C viable cells and vaccinia virus can be demonstrated in cultures composed of Tyrode's solution and chick embryonic tissue during about 4 weeks following inoculation. These results are contrasted with those previously obtained in roller tube cultures where survival of both cells and virus persists much longer and the multiplication of the virus is greatly increased.

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[8] Plotz, H., and Ephrussi, B., *Compt. rend. Soc. Biol.*, 1933, **112**, 525.

13497 P

Observations upon a Long-Surviving Cat with a Gross Bilateral Lesion in the Cervical Spinal Cord.

Asher E. Treat. (Introduced by W. S. Root.)

From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York, N. Y.

During the course of experiments previously reported[1] a male cat was prepared with an extensive bilateral lesion probably involving the left side of the eighth cervical segment and the right side of the first thoracic segment of the spinal cord. The lesion was produced on March 26, 1940. At the time of writing, 21 months after the operation, the animal appears to be in excellent condition. Its weight remains constant at about 3.04 kg, which is slightly above the preoperative value. There have been no pressure ulcers. The appetite is good, and although neither micturition nor defecation occurs spontaneously, the output of urine and feces is normal in quantity. Active spermatozoa are produced and are ejaculated in response to appropriate stimulation. Sensory anesthesia and volitional paralysis are apparent below the level of the lesion. Breathing is entirely diaphragmatic. The functional signs are those of complete interruption of the spinal pathways.

In addition to the usual spinal reflexes, which appear in this animal with regularity and vigor, there is occasionally exhibited a mass reflex of exceptional character which I have not seen described for laboratory animals. It is often elicited by rubbing the shoulder region in such a way as to evoke the typical scratch reflex. Upon continuation or intensification of the stimulus, the scratch response gives way to a violent flexor spasm of the muscles of the abdomen, which brings the hind feet into a position forward of the head, with the legs slightly abducted. As the flexor spasm begins, the rubbing of the shoulder region is discontinued, and the subsequent events occur without further external stimulation. For 5 to 10 seconds, the hind legs perform rapid clonic movements of small amplitude but of great force, while the distal part of the tail exhibits a lashing motion. During this time, if the bladder has been distended, there is a discharge of urine. Erection of the penis and ejaculation of semen may occur. The flexor spasm is abruptly followed by a series of running movements, lasting another 5 to 10 seconds, and involving alternate flexions and extensions both of the trunk and of the hind legs. This phasic activity gradually subsides, and for a minute or more the tail continues to twitch, and the hind legs display feeble clonic movements which appear and reappear like echos of the preceding stages. If the cat is examined during this time, it is found that the pupils are widely dilated, and that the pulse and respiration are increased in rate. The convulsion has rarely been evoked twice within a single day. Several weeks may elapse during which it is not obtained at all.

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[1] Treat, A. E., *Am. J. Physiol.*, 1941, **134**, 310.

13498 P

Prophylactic Value of Refined Antipertussis Rabbit Serum.*

WILLIAM L. BRADFORD, HENRY W. SCHERP AND ANN M. BROOKS.
From the Departments of Pediatrics and Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The globulin fraction of the serum of rabbits, immunized with living *Hemophilus pertussis,* was shown to protect mice against experimental infection with this organism.[1] The intramuscular injection of doses of from 4 to 7 ml of the refined serum into infants, in the early stage of whooping cough, produced an immediate increase in both the mouse-protective and agglutinative titers of the infants' sera.[2] In certain instances, there seemed to be a beneficial effect upon the clinical course of the disease, and frequently a definite decrease was noted in the total number of mononuclear cells of the blood.

The present report concerns the prophylactic use of the antiserum[†] in 20 infants exposed to pertussis by family contact.

When an older sibling was found to have whooping cough, the exposed infant was given an intramuscular injection of from 2 to 10 ml of the concentrated antiserum (after preliminary testing for sensitivity). The exposed infant then remained at home in contact with one or more infected siblings, and was observed carefully by clinical examination. In 13 cases the presence of pertussis in the family was confirmed by nasopharyngeal culture.

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[*] This work was aided in part by a grant from the John and Mary R. Markle Foundation.

[1] Scherp, H. W., Bradford, W. L., and Wold, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **43**, 172.

[2] Bradford, W. L., Scherp, H. W., and Brooks, A. M., *Am. J. Dis. Ch.*, 1941, **62**, 492.

[†] The concentrated antiserum was supplied through the courtesy of Dr. W. E. Bunney of E. R. Squibb & Sons. The methods of preparation and standardization have been briefly described.[2]

In the group of 20 infants 13 (65%) were completely protected. Of the 7 infants who developed pertussis after receiving the serum, however, 5 experienced mild cases, whereas the disease was unmitigated in the other two. Furthermore, in 3 of these 7 instances, the infants began to cough 5, 5, and 4½ weeks respectively after the serum was injected (the time of onset was not ascertained in 3 cases). It is unlikely that a passive immunity would be at an adequate level after this interval, especially in the presence of a continuing exposure to infection.[3] In no case were there complications. No general reactions occurred as a result of receiving the serum, although 2 infants did develop urticaria at the site of the injection.

It is impossible to know whether a given infant in this series would have escaped infection without the serum, but it is generally agreed that the communicability rate is between 70 and 100% under the ordinary conditions of family exposure.[4] During a recent study[5] of a school outbreak in Rochester, 31 of 36 (86%) preschool children exposed in families developed the disease. If the rate were 80%, the expected number of "escapes" in the present series would have been 4 (20%). Actually, 13 (65%) escaped infection. The difference (45%) is 3.2 times its standard error (14%) and therefore is statistically significant.

Auxiliary tests of the serum used in the present study indicate that its action is chiefly antibacterial rather than antitoxic. The relative importance of the antitoxic and antibacterial qualities of immune pertussal serum is not as yet entirely known, but recent work[6] suggests that antibacterial antibodies are more closely related to resistance. If this be true, it would seem that a more extended test of the value of an antiserum, such as the one discussed here, deserved attention.

The authors are indebted to Doctors John Aikman, Burtis Breese, Fred Bush, Michael Gerbasi, Albert D. Kaiser and other members of our Pediatric staff for their interest and coöperation in this preliminary study.

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[3] Topley, W. W. C., and Wilson, G. S., *The Principles of Bacteriology and Immunity*, Second Edition, William Wood & Company, Baltimore, 1936, p. 882.

[4] a. Von Sholly, A. I., Blum, J., and Smith, L., *J. A. M. A.*, 1917, **68**, 1451; b. Kendrick, P., and Eldering, G., *Am. J. Hyg.*, 1939, **29**, 133; c. Cullotta, C. E., Dominick, D., and Hanson, E. R., *Yale J. Biol. and Med.*, 1938, **10**, 473; d. Doull, J. A., Shibley, G. S., Haskins, G. E., Bancroft, H., McClelland, J. E., and Hoebscher, H., *Am. J. Dis. Ch.*, 1939, **58**, 691; e. Miller, J. J., and Faber, H. K., *Am. J. Dis. Ch.*, 1940, **60**, 1172.

[5] Clark, A. J., and Bradford, W. L., to be published.

[6] Flosdorf, E. W., McGuinness, A. C., Kimball, A. C., and Armstrong, J. G., *J. Ped.*, 1941, **19**, 638.

13499

Action of Aminoacetic Acid in Preventing Convulsions Produced by Metrazol.

LEWIS J. POLLOCK, ISIDORE FINKELMAN AND ELI L. TIGAY.*

From the Department of Nervous and Mental Diseases, Northwestern University Medical School.

In searching for another anticonvulsant that will not at the same time produce a narcotic effect, we have studied the effect of previous administration of aminoacetic acid upon the convulsions usually following the injection of metrazol in the rabbit. For the purpose of study, the severity of the observed attacks was defined and classified numerically as follows: Only a hunching backward as type 1; twitching of ears and head as type 2; myoclonic jerking of the muscles of the head, neck and extremities, the animal remaining erect as type 3; a more severe myoclonic jerking of these parts, the animal remaining erect as type 4; and a complete clonic-tonic-clonic convulsion, the animal falling to the ground as type 5.

As control experiments we induced convulsions in 100 rabbits by means of intravenous injection of metrazol. The minimal convulsant dose was found to be 13.2 mg of metrazol given intravenously per kilo of body weight of rabbit. After administration

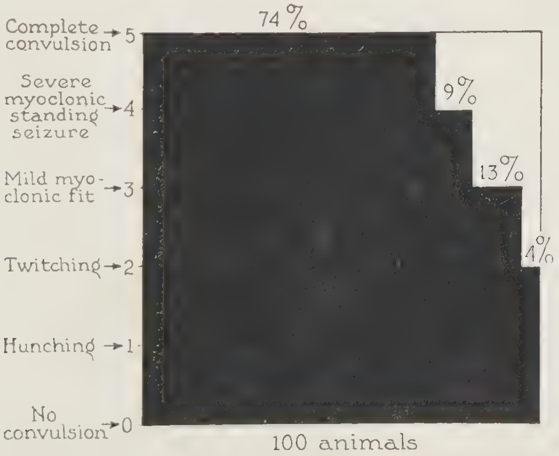

FIG. 1.

Percent distribution of degree of severity of convulsions in 100 animals which received an intravenous injection of 13.2 mg metrazol per kilo.

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* Acknowledgment is made to Bilhuber Knoll Corporation for furnishing the metrazol used.

of this dose, there ensued 74 (74%) complete convulsions (type 5), severe myoclonic standing seizures (type 4) in 9 (9%), 13 (13%) mild myoclonic standing seizures (type 3) and in 4 (4%) only twitchings (type 2). Fig. 1.

In 43 rabbits, 220 to 880 mg of aminoacetic acid per kilo weight were injected intravenously 5 minutes before the intravenous administration of a minimal convulsant dose of metrazol. Some protective action against convulsions produced by metrazol was found. In this group the injection of metrazol produced 53.5% of complete convulsions (type 5), 16.2% of mild myoclonic seizures (type 3), 11.6% of only twitches (type 2), 2.3% of hunching (type 1), and in 16.2% no motor disturbance was observed. Fig. 2. However, a great variability was seen in the action of aminoacetic acid in different groups of animals. Although aminoacetic acid prevented attacks in a significant number of instances and diminished the severity of attacks when they occurred, its anticonvulsant property is not as marked as other agents studied by us, such as pyridine, nicotinamide, some other pyridine derivatives and phenylcinchoninic acid.[1]

When metrazol was administered subcutaneously, the convulsion appeared only after a certain length of time sufficient for the absorption of a convulsive dose; in these cases previous injection of aminoacetic acid appears to be more effective in preventing convulsions than in these instances where metrazol is given intravenously.

The responses of rabbits to the subcutaneous injection of convulsive doses of metrazol shows a greater variation than those

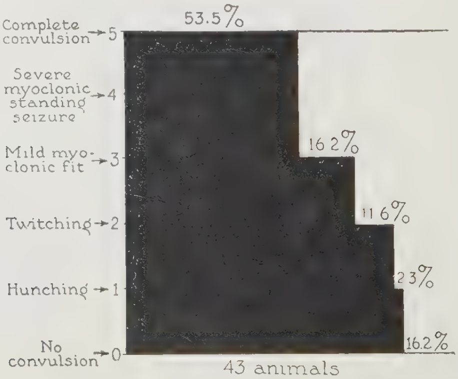

FIG. 2.

Percent distribution of degree of severity of convulsions in 43 animals which received 220 to 880 mg per kilo of aminoacetic acid intravenously 5 min. previous to the injection of a minimal convulsant dose of metrazol.

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[1] Pollock, L. J., Finkelman, I., and Tigay, E., to be published.

observed after intravenous injection of the same agent. A number of seconds after the intravenous administration of a minimal convulsant dose of metrazol there usually appears a single clonic-tonic-clonic convulsion. However, the subcutaneous injection of a convulsant dose of metrazol (36.5 to 55 mg per kilo of body weight) is followed by phenomena of a different nature.

Here, in about 60 seconds after injection, the animal becomes restless, raises the ears, hunches backward, sits on the hind legs, shakes the head and appears frightened. During this period the respirations become rapid and deep. These are followed by a period in which the animal shivers, becomes rigid and at times ataxic. Twitching and myoclonic jerking appear at different time intervals after injection; in some these occur as early as 2 minutes after injection; in others up to 19 minutes. In some a complete convulsion follows the twitching and myoclonic jerking; in others a seizure of lesser intensity may ensue and these milder seizures may be repeated at intervals of from 2 to 31 minutes. When the first seizure is complete it may appear as early as 2 minutes after injection and as late as 27 minutes afterward. Usually these severe seizures are repeated at intervals of an hour or more, and in a few instances these seizures occurred one after another as in a status.

Schoen,[2] Camp,[3] and Lendle[4] stated that the convulsive dose of metrazol administered subcutaneously is 50 mg per kilo of body weight. Hildebrandt and Voss[5] and Schubel and Gehlen[6] place this dosage at 40 mg and Hildebrandt and Mugge[7] concluded after a large number of experiments that 35 mg is sufficient.

In our experiments with a group of 28 rabbits used as a control (Fig. 3A) the subcutaneous injection of from 36.5 to 55 mg of metrazol per kilo of body weight produced, after an interval of varying length, a complete convulsion (type 5), in 39.3%, a severe standing myoclonic seizure (type 4) in 7.1%, a mild standing myoclonic seizure (type 3) in 4.3%, twitches only (type 2) in 17.8%. No motor manifestations were seen in 21.4%.

Five minutes before the subcutaneous administration of a convulsant dose of metrazol, 25 animals were given aminoacetic acid intravenously in doses of from 330 to 440 mg per kilo of body weight. In these, there resulted (Fig. 3B) a complete convulsion

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[2] Schoen, R., *Arch. f. exp. Path. u. Pharm.*, 1926, **113**, 257.

[3] Camp, W. J. R., *J. Pharm. and Exp. Therap.*, 1928, **33**, 81.

[4] Lendle, L., *Arch. f. exp. Path. u. Pharm.*, 1936, **181**, 408.

[5] Hildebrandt, F., and Voss, J., *Munch. Med. Wschr.*, 1926, **73**, 862.

[6] Schubel, K., and Gehlen, W., *Arch. f. exp. Path. u. Pharm.*, 1928, **133**, 295.

[7] Hildebrandt, F., and Mugge, H., *Schwerz Narkose—Anaesthesie*, 1936, **9**, 25.

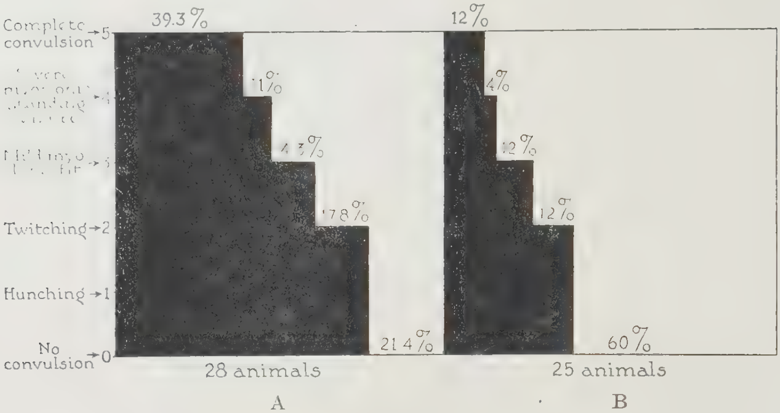

FIG. 3A.

Percent distribution of degree of severity of convulsions in 28 animals which received a subcutaneous injection of metrazol, 36.5 to 55 mg per kilo.

FIG. 3B.

Percent distribution of degree of severity of convulsions in 25 animals which received 330 to 440 mg of aminoacetic acid per kilo 5 min. before the subcutaneous injection of 36.5 to 55 mg per kilo of metrazol.

(type 5) in 12%, a severe standing myoclonic seizure (type 4) in 4%, a mild standing myoclonic seizure (type 3) in 12%. In 12% only twitches (type 2) were observed and 60% showed no motor manifestations.

Most significant is the observation that in the group treated with aminoacetic acid 12% had complete convulsions induced by metrazol, while in the group not so treated, 39.3% had complete convulsions. Further, in the treated group 60% showed no motor manifestations, while in the untreated group this ratio was 21.4%.

We have not been able to determine the reason for this anticonvulsant property. Aminoacetic acid as well as glucuronic acid contributes significantly to the detoxication of many substances by conjugation as for example Borneol. It may be possible that aminoacetic acid acts as a detoxicant when metrazol is injected subcutaneously and thereby diminishes the severity of and at times prevents the convulsions often seen after such injections.

Conclusions. Aminoacetic acid affords some protection against convulsions produced by the intravenous injection of metrazol. It is insignificant as compared to other substances, as pyridine, nicotinamide and phenylcinchoninic acid. Aminoacetic acid affords a great deal of protection against convulsions produced by the subcutaneous injection of metrazol.

13500

Effects of Tyrothricin and Actinomycin A Upon Bacterial Fibrinolysis and Plasma-Coagulation.

Erwin Neter.

From the Bacteriological Laboratories of the Children's Hospital and the Department of Bacteriology and Immunology, University of Buffalo, School of Medicine, Buffalo, N. Y.

Antagonism between various microörganisms has been known for many years. Recently, these phenomena have again attained attention in an effort to determine their mechanisms and possible applications for the prevention and treatment of infectious diseases. Antagonistic interrelations of microörganisms have been admirably reviewed by Waksman.[1] Of particular interest are antagonistic phenomena due to the production and liberation of bacteriostatic or bactericidal substances. Fleming,[2] Abraham and associates[3] obtained a substance from *Penicillium notatum,* referred to as penicillin, that is markedly antagonistic to certain microörganisms. Waksman[4] succeeded in isolating from *A. antibioticus 2* such substances, that were designated as actinomycin A and B. The former exerts highly selected bacteriostatic effects, whereas the latter is largely bactericidal. Dubos and Hotchkiss[5] isolated from *Bacillus brevis 2* substances with marked antimicrobial activity: Gramicidin, acting upon gram-positive bacteria, and tyrocidin, which affects both gram-positive as well as gram-negative bacteria. Tyrothricin is the product obtained from *Bacillus brevis* that contains both gramicidin and tyrocidin.

In view of the marked antagonistic activity of these substances of microbial origin toward many bacteria and fungi the question arises as to whether or not they act also upon bacterial toxins and toxin-like substances. In the following communication experiments are presented dealing with the effects of tyrothricin and actinomycin A upon fibrinolysis by *beta hemolytic streptococcus* and plasma-coagulation by staphylococcus.

Tyrothricin was obtained through the courtesy of Dr. D. F.

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[1] Waksman, S. A., *Bact. Rev.*, 1941, **5**, 231.

[2] Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 226.

[3] Waksman, S. A., and Woodruff, H. B., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 609.

[4] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Florey, H. W., Heatley, N. G., and Jennings, M. A., *Lancet*, 1941, 177.

[5] Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, 1941, **73**, 629.

Robertson, Associate Medical Director, Merck & Company. A stock solution was prepared by dissolving 200 mg of tyrothricin in 10 cc of 95% ethyl alcohol. Appropriate amounts of this alcoholic solution were added to physiological saline solution to give the desired concentrations of the drug.

Actinomycin A was made available through the kindness of Dr. Selman A. Waksman, State of New Jersey Agricultural Experiment Station, New Brunswick, N. J. A stock solution was prepared by dissolving 10 mg of actinomycin A in 10 cc of 95% ethyl alcohol. Adequate amounts of this stock solution were dissolved in physiological saline solution to obtain the desired concentrations.

In the tests on fibrinolysis the technic of Tillett and Garner[6] was followed. Two strains of *beta hemolytic streptococcus* (Group A) obtained from man were used as source of fibrinolysin. Partially purified fibrinolysin was obtained through the courtesy of Dr. W. S. Tillett and Dr. L. R. Christensen. The technic of the staphylococcal coagulase test was essentially the same as that described by Fisk.[7] A strain of *Staphylococcus aureus hemolyticus* obtained from a skin lesion was used as source of coagulase.

Plasma was obtained from human sources. One cc of a 2% potassium oxalate solution was evaporated in small bottles. Five cc of human blood were added and shaken well. Plasma was removed following centrifugalization of the blood.

In order to determine the effects of tyrothricin and actinomycin A upon the fibrinolytic activity of *beta hemolytic streptococcus* (Group A Lancefield) the following experiment was carried out. The supernatant of an 18-hour infusion broth culture of the streptococcus (volume 0.5 cc) in a dilution of 1:10 was mixed with various amounts (volume 0.5 cc) of tyrothricin (0.01 mg to 0.000001 mg) and actinomycin A (0.0005 mg to 0.0000005 mg), respectively. As controls, the supernatant of the streptococcus culture was mixed with physiological saline solution and saline solution containing 0.95% alcohol, respectively. The latter control was included in order to determine whether small amounts of alcohol, as present in the tyrothricin and actinomycin A solutions, affect fibrinolysis. To these mixtures 0.2 cc of human plasma and 0.25 cc of an 0.25% calcium chloride solution were added. The tubes were incubated at 37°C. The resulting clot formation and secondary lysis of the plasma clots were noted. The results of this experiment are summarized in Table I.

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[6] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

[7] Fisk, A., *Brit. J. Exp. Path.*, 1940, **21**, 311.

TABLE I.

Effects of Tyrothricin and Actinomycin A upon Fibrinolysis by *Streptococcus hemolyticus.*

		Supernatant of streptococcal culture (volume 0.5 cc) diluted 1:10 Plasma (volume 0.2 cc) $CaCl_2$ 0.25% (volume 0.25 cc) Fibrinolysis following incubation at 37° C for		
		10 min.	3 hr	24 hr
Tyrothricin in mg (vol. 0.5 cc)				
1.	.01	—	—	—
2.	.001	—	—	—
3.	.0001	—	++++	++++
4.	.000001	—	++++	++++
5.		—	++++	++++
Actinomycin A in mg (vol. 0.5 cc)				
1.	.0005	—	—	—
2.	.00005	—	—	—
3.	.000005	—	++++	++++
4.	.0000005	—	++++	++++
5.		—	++++	++++
Alcohol Control		—	++++	++++

— = No fibrinolysis (clot formation).
+ to ++++ = Various degrees of fibrinolysis.

It may be seen from Table I that (1) fibrinolysis occurred in the controls after an incubation period of 3 hours and that (2) fibrinolysis was completely and continuously prevented by both tyrothricin and actinomycin A. Essentially the same results were obtained in several experiments employing two different strains of streptococci. It may be mentioned, however, that the degree of inhibition of fibrinolysis varied somewhat with different plasmas employed. In some experiments inhibition of fibrinolysis was only transitory.

The inhibitory effects of tyrothricin and actinomycin A upon fibrinolysis by streptococcus was noted also when tyrothricin was allowed to act for several hours at 4°C upon the streptococcal culture or supernatant thereof. No marked increase in the inhibitory effects were observed.

Controls indicated that neither tyrothricin nor actinomycin A in the amounts used caused either clotting of plasma or lysis of plasma clots.

The problem arises as to whether or not this inhibition of streptococcal fibrinolysis is due to a direct action of tyrothricin and actinomycin A upon the fibrinolysin itself. No final answer can as yet be given. A preliminary experiment with partially purified fibrinolysin failed to reveal any definite inhibitory effects.

It is interesting to mention that according to Doudoroff[8] certain bacteria exert antifibrinolytic activity, among them, certain streptococci, *Proteus vulgaris, Ps. fluorescens, Ps. aeruginosa, B. anthracis, Cl. sporogenes,* and *Cl. histolyticum.* The question presents itself as to whether this antifibrinolytic activity is due to chemical substances produced and liberated by these microörganisms, and, furthermore, whether other microörganisms, pathogenic or non-pathogenic, also interfere with streptococcal fibrinolysis.

The effects of tyrothricin and actinomycin A upon plasma-coagulation by *Staphylococcus aureus hemolyticus* were investigated in the following experiment. Tyrothricin in amounts ranging from 0.01 mg to 0.00001 mg and actinomycin A in amounts of 0.0005 mg to 0.0000005 mg (volume 0.5 cc) were mixed with 0.2 cc of a 1:10 diluted 18-hour broth culture of the staphylococcus. To this mixture 0.5 cc of 1:5 diluted human plasma was added. The tubes were shaken and incubated at 37°C for 18 hours. The resulting clot formation was noted at various intervals. The results of this experiment are summarized in Table II.

It may be seen from Table II that (1) *Staphylococcus aureus hemolyticus* culture caused coagulation of human plasma after an

TABLE II.
Effects of Tyrothricin and Actinomycin A upon Plasma Coagulation by *Staphylococcus aureus hemolyticus.*

		Staphylococcal broth culture (volume 0.2 cc) dilution of 1:10 Plasma (volume 0.5 cc) in dilution of 1:5 Clot formation after incubation at 37°C for			
		1½ hr	2½ hr	4 hr	18 hr
	Tyrothricin in mg (vol. 0.5 cc)				
1.	.01	—	—	—	—
2.	.001	—	—	—	+
3.	.0001	—	—	+	+
4.	.00001	+	+	+	+
5.		+	+	+	+
	Actinomycin A in mg (vol. 0.5 cc)				
1.	.0005	—	—	—	—
2.	.00005	+	+	+	+
3.	.000005	+	+	+	+
4.	.0000005	+	+	+	+
5.		+	+	+	+
Alcohol control		+	+	+	+

+ = Clot formation.
— = No clot formation.

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[8] Doudoroff, M., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1467.

incubation period of 90 minutes; (2) in contrast, clot formation did not occur in the presence of tyrothricin and actinomycin A. The inhibitory effect of tyrothricin and actinomycin A upon plasma-coagulation by *Staphylococcus aureus hemolyticus* could be demonstrated in repeated experiments. These substances also prevented clot formation by supernatants of staphylococcal cultures. Furthermore, this inhibitory effect of tyrothricin and actinomycin A upon plasma-coagulation by staphylococci was observed also when the mixtures were incubated for 18 hours at either 37°C or at 4°C prior to the addition of plasma.

It may be mentioned that neither tyrothricin nor actinomycin A in the above amounts prevented coagulation of human plasma following the addition of calcium chloride; furthermore, that the addition of calcium chloride to mixtures of staphylococcal culture, human plasma and tyrothricin or actinomycin A, that failed to show coagulation of plasma, resulted in prompt clot formation. As yet, it cannot be stated whether or not this inhibitory effect of tyrothricin and actinomycin A upon plasma-coagulation of staphylococcus is due to a direct action upon the coagulase.

There is experimental evidence indicating that fibrinolysin and coagulase play a rôle in the pathogenesis of streptococcal and staphylococcal infections. It remains to be determined whether tyrothricin and actinomycin A inhibit fibrinolysis and plasma-coagulation also *in vivo* and what significance, if any, this inhibitory effect has upon the course of the infection.

Summary. (1) Tyrothricin and actinomycin A inhibit fibrinolysis by *beta hemolytic streptococcus* cultures or supernatants thereof. (2) These substances also interfere with coagulation of plasma by pathogenic staphylococcus.

13501

Improved Mechanical Microtome Knife Sharpener.

FRANK W. HARTMAN, JR., AND F. W. HARTMAN.

From the Department of Laboratories, Henry Ford Hospital, Detroit, Mich.

For more than ten years the automatic microtome knife sharpener described by Fanz[1] has been standard equipment in most large pathology laboratories, saving many hours of tedious labor while producing a far better cutting edge on the knives than hand work could possibly do.

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[1] Fanz, J. I., *J. Lab. and Clin. Med.*, 1929, **14**, 1194.

In the apparatus presented here, the glass plate described by Minot and Fanz has been incorporated but the mechanics have been simplified. The time needed for sharpening has been reduced by swinging the knife through an ellipse rather than an arc alone.

Description of Machine. The whole apparatus (Fig. 1) is mounted on a pedestal which has a heavy base fitted with 2-inch casters. P is a 1/20 H.P. geared motor with a ¾-inch shaft, 4 inches in length, presenting vertically. This shaft carries the small gear I and the 8-inch disc C. The disc C in turn mounts the three 3-inch vacuum cups on which rests the 24-inch glass plate B. This type of mounting holds the plate securely in place but absorbs the torque of starting and stopping. The larger 10-inch gear J is mounted on the supporting plate and attached to it is one of the flat bearings which, with its mate, holds the carriage K for the knife F and turning mechanism G-H. In the center of each bearing is a socket for a locking pin which, when placed in the bearing on J rotates the knife through an ellipse, and when placed in the second bearing rotates the knife through an arc only. The knife may be held fixed in any position desired on the plate by disengaging the small gear I with the lever O.

Mounted on the knife carriage K is the fixture bearing the small motor and turning mechanism H as well as the knife arm and

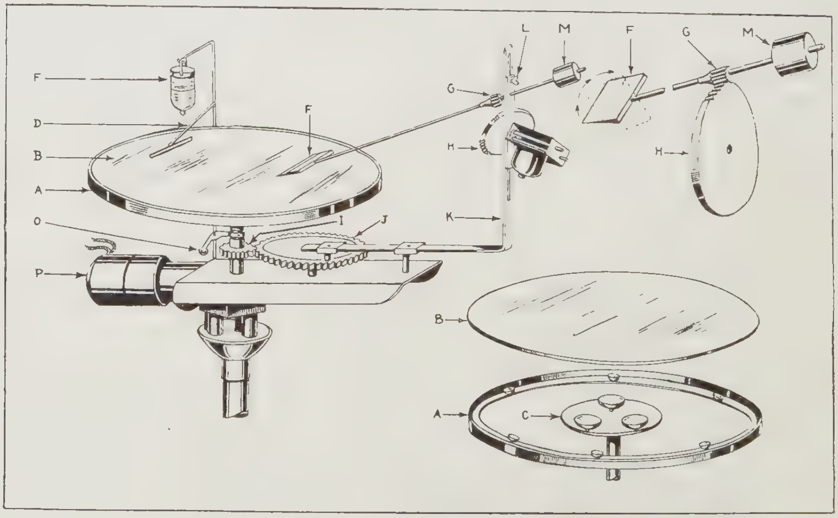

FIG. 1.

counter weight M. This fixture is attached with set screw L and hence is adjustable for any knife bevel without disturbing the relation of the turning gears G and H. H, an elliptical fiber gear, is actuated by a small geared motor which runs continually; the butterfly gear turns H intermittently through one-sixth of the arc every 15 seconds. Hence the knife is turned over every 90 seconds and the actual rotation takes only 5 seconds so that little time is lost from the sharpening procedure.

The drip pan is of light galvanized material and is attached to the under-surface of the glass plate by 6 small vacuum cups.

The solutions are supplied to the plate by a drip bottle F. The bottle support also carries a "Mercury" windshield wiper assembly D, including the spring tension which makes for efficiency and avoids unnecessary dragging on the plate.

The machine operates smoothly and very quietly. The knife is turned quickly and without effort. Little attention from the technician is necessary except for the changing of the knife and various grinding compounds. Simplification of the apparatus reduces the cost of construction to a range well within the means of the smaller laboratory.

13502

Effect of Phosphoric Acid Esters on Fracture Healing.*

W. D. ARMSTRONG, LOUIS SPERLING AND SIDNEY LITOW.

From the Division of Physiological Chemistry and the Department of Surgery, University of Minnesota, Minneapolis, Minn.

The erythrocytes contain an abundance of phosphoric esters hydrolyzable by phosphatase,[1] but the plasma contains only 0.2-0.5 mg phosphorus per 100 cc[2] in the form of phosphatase hydrolyzable esters. The phosphatase activity of a fracture callus increases rapidly beginning 3-4 days after the injury.[3, 4, 5] Although the site

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* This work was supported by Grant 557 of the Committee on Scientific Research of the American Medical Association.

[1] Kay, H. D., and Robison, R., *Biochem. J.*, 1924, **18**, 755.

[2] Martland, M., and Robison, R., *Biochem. J.*, 1926, **20**, 847.

[3] Wilkins, W. E., and Regen, E., PROC. SOC. EXP. BIOL. AND MED., 1924, **32**, 1373.

[4] Botterell, E. H., and King, E. J., *The Lancet*, 1935, **7**, 1267.

[5] Tollman, J. P., Drummon, D. H., McIntyre, A. R., and Bisgard, J. D., *Arch. Surg.*, 1940, **40**, 43.

of an old fibrous non-united fracture may exhibit low phosphatase activity[3] and infected fractures contain somewhat reduced amounts of this enzyme[6] there is no evidence that defective calcification of bone or delay in fracture healing is primarily a result of a quantitative deficiency of this enzyme. It appears possible that one of the factors limiting the speed of the calcification process is the low content of plasma with respect to the substrate of bone phosphatase and this consideration suggested that some cases of delayed or nonunion of fractures might be so explained. It seemed desirable, therefore, to investigate the effect of the intravenous injection of sodium beta-glycerophosphate on the healing of fresh fractures.

The right radius of rabbits, weighing between 4.25 and 4.75 lb, was fractured while the animals were anesthetized. The fractures were produced by grasping the middle of the shaft of the bone between two adjacent and parallel Kocher forceps and exerting steady rotation of the forceps in opposite direction until the bone was heard to break. A few animals, in which breaks of both the radius and ulna were produced, or which displayed marked bruising of the skin, were discarded. Roentgenological examination showed that the fractures usually were transverse and that the fragments were in good alignment with little comminution of the bone. The undamaged ulna served as an effective splint and the animals were able to use the limb upon recovery from the anesthesia.

Twenty-seven animals of the experimental group received 6-14 daily injections, by ear vein, of 0.1 g sodium beta-glycerophosphate in 4 cc of solution. Twenty-two animals with similar fractures served as controls and received a like number of injections of 4 cc of physiological saline. All animals were sacrificed on the day following the last injection. Roentgenograms of the limbs, taken before and after removal of the soft tissues, did not reveal any marked differences except for a larger callus about the fracture of the bones of the animals treated with sodium beta-glycerophosphate. However, the fracture callus of the animals receiving 8-14 treatments with sodium beta-glycerophosphate was larger and more firm to palpation than that of the control animals. After the bones were cleaned, even though some of the callus was lost when the periosteum was removed, the healing of the fracture appeared to have progressed further in the animals treated with glycerophosphate. This judgment was based upon the observation that the hard callus of the treated animals was larger and the fracture defect was more nearly bridged over in the treated animals than in the controls. A more objective means of estimating the degree of healing of fractures would be desirable. However, the observations indicated that in the majority of cases the drug exerted a positive effect even though the fractures with which we were dealing normally progress to rapid healing. Four determinations of serum inorganic phosphate and calcium, made at regular intervals during the course of 12 daily injections of 0.1 g of glycerophosphate, showed no consistent differences from the results obtained with untreated animals.

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[6] Drummond, D. H., Tollman, J. P., Bisgard, J. D., and Ross, D., *Arch. Surg.*, 1940, **40**, 49.

Sodium beta-glycerophosphate, when given by intravenous injection over a long time or in large doses, is not toxic. The liver, intestine, urinary bladder, kidney, heart and aorta of a rabbit which received 55 daily injections of 0.1 g of the drug were normal grossly and microscopically. Similar results were obtained with 3 rabbits following 6 daily intravenous injections of 1.0 g of the substance. No symptoms were produced following 26 daily subcutaneous injections of 0.1 g of sodium beta-glycerophosphate into a rabbit and no pathological findings were noted at autopsy. A 17 kg dog received 10 daily intravenous injections of 10 g of the substance in 40 cc of solution. No ill effects were produced and when the animal was sacrificed, 2 months after the last injection, and no pathological alterations of any organ were observed. Large doses (12 to 24 cc of a solution containing 0.25 g of the substance per cc) are fatal in 2-5 days when given daily to rabbits by intraperitoneal injection.

Since sodium beta-glycerophosphate is innoxious in a wide range of dosage it should be safe to administer it to the human in order to determine its effect on those fractures which heal at a rate slower than usual.

13503

Activity of Certain Isomers of Thyroxin.*

Paul D. Boyer, Curtis W. Jensen and Paul H. Phillips.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Investigations on compounds closely related structurally to thyroxin have been limited. An isomer of thyroxin, β-β-di (3,5-diiodo-

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4-hydroxyphenyl) phenylalanine, has been synthesized by Harington and McCartney,[1] and found to be inactive. Thyronine has been found inactive,[2, 3] and diiodothyronine has been shown to have slight activity.[3, 4] Recently Nieman and coworkers[5, 6] have synthesized two isomers of thyroxin differing from thyroxin only in the position of the hydroxyl group and iodine atoms in the second benzene ring.[†] These experiments were undertaken to determine the effect of these compounds on the basal metabolic rate of the rat.

The first isomer studied was dl-3,5-diiodo-4-(2′-4′-diiodo-3′-hydroxyphenoxy)-phenylalanine (II) which can be more conveniently termed "meta" thyroxin on the basis of the position of the hydroxyl group, ordinary thyroxin (I) being the para compound. As this compound was only available in limited quantity, studies on possible toxic effects and preliminary studies on the effect on the basal metabolic rate were made at the same time. The methods used for determination of the B.M.R. were given in an earlier publication.[7] The meta thyroxin was dissolved in a minimum amount of sodium hydroxide, and injected subcutaneously at a level of 5 mg per kg of body weight, using a rat whose B.M.R. had previously been standardized. Since the B.M.R. determinations indicated that this level was ineffective, subsequent doses of 30, 60, 120, and 180 mg per kg of body weight were given over a period of 6 days, all without any toxic effect or without any definite effect on the B.M.R. To test larger amounts, doses of 250 and 500 mg per kg of body weight were given to 2 rats whose B.M.R. had been previously standardized. These doses had little or no effect as the B.M.R. remained at a normal level of about 1000 calories per square meter per 24 hrs. In contrast to the lack of effect of the meta thyroxin, synthetic dl-thyroxin in doses of 1 mg per kg resulted in an increase of 25-35% in the B.M.R.

Preliminary studies with "ortho" thyroxin, dl-3,5-diiodo-4-

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[1] Harington, C. R., and McCartney, W., *J. Chem. Soc.*, 1929, 892-7.

[2] Gaddum, J. H., *J. Physiol.*, 1930, **68**, 383.

[3] Thompson, W. O., Thompson, P. K., Taylor, S. G., III, Nadler, S. B., and Dickie, L. F. N., *Endocrinology*, 1935, **19**, 14.

[4] Rapport, D., and Canzanelli, A., *Am. J. Physiol.*, 1933, **103**, 279.

[5] Niemann, C., and Redemann, C. E., *J. Am. Chem. Soc.*, 1941, **63**, 1549.

[6] Niemann, C., and Mead, J. F., *J. Am. Chem. Soc.*, 1941, **63**, 2685.

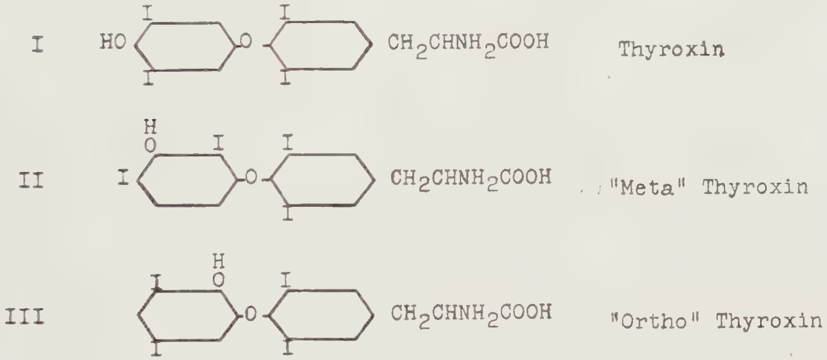
[†] These compounds have been made available to us by Dr. Carl Niemann, of the California Institute of Technology, with whom this was a coöperative research project.

[7] Boyer, P. D., Evans, R. J., and Phillips, P. H., *J. Pharm. and Exp. Therap.*, 1941, **73**, 176.

TABLE I.
Effect of "Ortho" Thyroxin* on B.M.R. of Thyroidectomized Rat.

Days after injection	Calories per sq m per 24 hrs		
	Rat No. 3 25 mg/kg	Rat No. 4 25 mg/kg	Rat No. 5 50 mg/kg
Thyroidectomized B.M.R.	620	880	740
4	950	1100	940
11	740	1030	790
18	640	930	690
25	650	900	690

*dl-3,5-diiodo-4-(3′,-5′-diiodo-2′-hydroxyphenoxy)-phenylalanine.



Formulae

(3′-5′-diiodo-2′-hydroxyphenoxy)-phenylalanine (III), indicated that this compound had some biological activity. Accordingly, studies were made using thyroidectomized rats. A test was not considered successful unless the thyroidectomized animal had a stable lowered B.M.R. both before and after showing a response to the test compound. The results showed that the ortho thyroxin possessed some activity in moderately large doses, as indicated in Table I. The responses were of the same magnitude as those given by synthetic dl-thyroxin in doses of 0.5-1.0 mg per kg of body weight. Thus the "ortho" thyroxin had an activity approximately 1/25-1/50 that of thyroxin. This compound is the only isomer of thyroxin which has been shown to have some biological activity. The activity of the ortho compound and lack of activity of the meta compound support the hypothesis advanced by Niemann[5, 6] that thyroxin-like activity is dependent upon the formation of a quinoid structure.

Summary. Two closely related isomers of thyroxin have been tested biologically. Meta thyroxin had little if any effect upon the

B.M.R. of the rat in doses up to 500 mg per kg of body weight, while ortho thyroxin was found to have an activity of 1/25 to 1/50 of that of thyroxin.

13504

Lysine Requirement of the Chick.

H. J. ALMQUIST AND E. MECCHI.

From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley.

In a continuation of studies on the quantitative amino acid requirements of the young chick for optimum rate of growth, evidence has been obtained on the level of lysine needed in the diet. The diet used was the same as that reported previously,[1] except for differences in the proteins and amino acids added. Calculations of amino acid levels in the diets are based upon the amino acid composition of proteins as summarized by Calvery.[2] Such calculations are, of course, subject to revision in the event that any of the cited values should be greatly altered in the future. The fact that lysine is required by the chick has been previously indicated.[3]

The initial experiment was conducted with zein since this protein contains no appreciable amount of lysine. After some failures in preliminary trials, the manifold deficiencies of zein were corrected by addition of pure amino acids and satisfactory rates of growth of

TABLE I.
Growth-promoting Effects of Lysine Additions to the Zein Chick Diet.

l-(+)-lysine added, %	Avg gain per chick, g	Gain per day, %
0.0	— 6.2	—1.7
0.4	+ 1.0	+0.2
0.8	+33.1	+6.4
Positive control diet	+40.7	+6.9

Each group contained 5 chicks with an average starting weight of 57 g. The experiment lasted 7 days. The positive control diet contained an equal weight of casein in place of zein. No methionine, threonine, tryptophane or lysine was added.

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[1] Almquist, H. J., and Mecchi, E., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 526.

[2] Calvery, H. O., *Chemistry of the Amino Acids and Proteins* (edited by C. L. A. Schmidt), Charles C. Thomas, publisher, Baltimore, Maryland, first ed., p. 217.

[3] Buckner, G. D., Peter, A. M., Wilkins, R. H., and Hooper, J. J., *Ky. Agr. Exp. Sta. Bul.*, 1919, **220**.

chicks were obtained. The final basal diet contained zein 30, arginine monohydrochloride 0.5, dl-methionine 0.2, dl-threonine 0.3, l-tryptophane 0.4, and glycine 1.0% as the sole sources of amino acids.

The results in Table I show that chicks receiving no lysine in the diet slowly lost weight, that 0.4% lysine in the diet was barely sufficient for maintenance of weight and that 0.8% lysine in the diet permitted a rate of growth which was slightly less than that of chicks receiving casein in place of zein (lysine level of diet approximately 1.8%).

Because of the necessity of many pure amino acid supplements to the zein diet and the corresponding high cost of this diet, experiments were next attempted with edestin, a protein which appeared complete for the chick in all respects except for lysine and possibly methionine. Edestin is known to be a good source of threonine[4] concerning the distribution of which almost no quantitative data are available.

Diets were prepared as before using as sources of amino acids, edestin 25, glycine 1.0, and dl-methionine 0.4%. The calculated lysine content of this diet was 0.55%. It was expected to be inadequate for optimum growth of the young chick, and so proved. The strict necessity of the methionine addition was not tested. It was not found necessary to add threonine, arginine or tryptophane. l-(+)-lysine monohydrochloride was added to the diet in amounts to adjust the total lysine content to the levels indicated in Table II. The results of this second experiment are in agreement with those of the first in showing that a level of more than 0.8% lysine was needed for the highest rate of growth. The results also suggest that the optimum level of lysine is probably not more than 0.9% in the diet.

In a third experiment, combinations of edestin with casein and with zein were used to provide various lysine levels in the diet, as

TABLE II.
Growth-promoting Effects of Lysine Additions to the Edestin Chick Diet.

Total l-(+)-lysine in diet, %	Avg gain per chick, g	Gain per day, %
0.55	+19.6	+3.3
0.65	+29.2	+4.7
0.75	+39.0	+5.9
0.85	+47.9	+6.7
0.95	+48.2	+6.8

Each group contained 5 chicks with an average starting weight of 55 g. The experiment lasted 10 days.

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[4] Rose, W. C., *Physiol. Rev.*, 1938, **18**, 109.

calculated from tables of amino acid composition of proteins.[2] Glycine was added where necessary to keep its level in the diet at 1% or more. Similarly, methionine (dl) was added to maintain a level of at least 0.75% of the natural form. Diet III-A, contained edestin 20 and casein 10%, diet III-B contained edestin 25 and casein 5%, diet III-C contained edestin 30%, and diet III-D contained edestin 25 and zein 5%. The calculated lysine levels in these diets are, respectively, 1.04, 0.85, 0.66, and 0.55%.

The results of the third experiment (Table III) also indicate that a maximum growth response would have been attained at approximately 0.9% lysine in the diet. Group III-D gained very slowly at the lowest level of lysine, but immediately accelerated to a nearly optimum rate of gain when lysine was added to the diet. This, as well as the results of the earlier experiments, indicates that the only growth-limiting deficiency in these diets, effective during the interval of the experiments, was that of lysine. The lysine level required for the maximum rate of growth of the young chick is approximately 0.9% of the diet. This level is indicated by calculations from tables of amino acid analyses, by additions of pure lysine to the diet and by both jointly.

Most of the amino acids used in this work were generously provided by Merck and Co., Inc.

Summary. The l-(+)-lysine requirement for optimum growth of the young chick is approximately 0.9% of the diet.

TABLE III.
Growth-promoting Effects of Various Levels of Lysine as Provided by Combinations of Proteins in the Chick Diet.

Group	Total l-(+)-lysine in diet, %	Avg gain per chick, g	Gain per day, %
III-A	1.04	+72.4	+7.0
III-B	0.85	+70.5	+6.9
III-C	0.66	+27.6	+3.4
III-D (1 to 8 days)	0.55	+ 8.4	+2.6
III-D (9 to 14 days)	0.85	+33.6	+6.5

Each group contained 5 chicks with an average starting weight of 57 g. The experiment lasted 11 days in the case of the first 3 groups.

After a period of slow growth for the first 8 days, Group III-D was given supplementary lysine in the diet, to a total level of 0.85%, and kept on this diet for 6 more days.

13505

A Photoelectrical Recorder for Biological Purposes.

SERGEI FEITELBERG.* (Introduced by Harry Sobotka.)
From the Laboratories of the Mount Sinai Hospital, New York.

The conventional method of manometric recording of blood pressure in animal experiments with a mercury manometer and a float, which carries a writing point, has been essentially the same since Ludwig described it in the last century. A vertical kymograph and specially treated paper (lamp black, etc.) are necessary, as any substantial friction between the paper surface and the writing point causes the float to submerge. This happens occasionally in spite of all precautions. A comparatively wide manometer tube has to be used in order to support the float, which has to carry the writing point and to overcome the friction on the paper; a wide tube draws several cc of blood from the vessels of the animal, when a rise in blood pressure occurs, and therefore a manometer cannot be used in small animals.

To eliminate these shortcomings of manometric recording an apparatus was designed where the float is substituted by a light beam (Fig. 1).

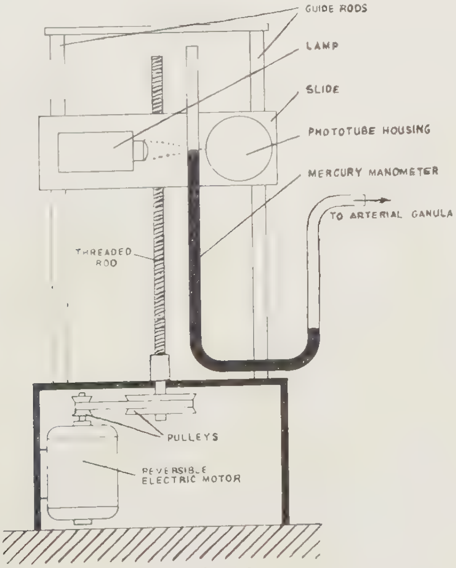

FIG. 1.
Schematic diagram of the recorder.

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* Aided by a grant from the Dazian Foundation for Medical Research.

A slide carries a lamp and a phototube. It has a female thread, which engages a threaded brass rod. This rod is rotated by a reversible electric motor. When the motor runs clockwise, the slide—and with it the lamp and the phototube—are raised, at a speed of 2 cm per second, and it is lowered at the same speed when the motor runs counterclockwise. After amplification† the phototube current operates a relay. The relay contacts are connected with the motor in such a way that the motor runs clockwise when the relay is open, and counterclockwise when it is closed.

When the light beam from the lamp strikes the phototube (the phototube housing has a hole 2 mm in diameter), the relay closes, the motor runs counterclockwise and the slide goes down, until the light beam is cut off by the mercury in the manometer tube. Then the relay opens, the motor is reversed and the slide goes up, until the light strikes the phototube again, and the cycle is repeated. This results in an oscillation of the slide at the level of the mercury meniscus; the stroke is 1 to 2 mm, the period ½ second. When the mercury level changes, the light beam, *i. e.,* the slide—phototube—lamp assembly, follows these changes. The maximal speed is 20 mm per second.

Fig. 2 illustrates a record of blood pressure, as obtained with this apparatus, with an ink-writer attached directly to the slide.

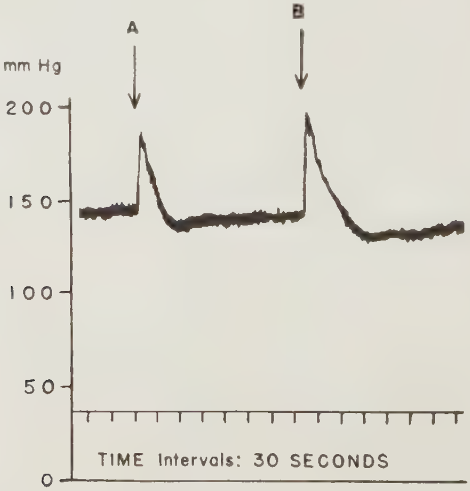

FIG. 2.

Record of arterial blood pressure with the photoelectrical recorder. Cat, 2.5 kg. At the arrows intravenous injection of adrenalin, at A—30 γ, at B—50 γ.

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† A conventional phototube amplifier is used. Blueprints of the electrical circuit will be sent by author upon request.

The limitations of the apparatus are determined by its mechanical characteristics. They come near to those of the mercury manometer itself. For a close analysis of the pulsewave, however, an inertia-free manometer and photographic recording are still necessary. The advantages of the photoelectrical recorder are as follows: 1. Any kind of recording can be used, as the power is supplied by the motor. An ink writer on a horizontal kymograph using a paper roll can be connected to the slide. 2. A very thin mercury column can be used; thus the amount of blood entering the manometer system upon a rise of blood pressure is substantially reduced. 3. The deflection of the manometer can be amplified by mechanical means (in the mechanical connection of the slide with the writer) and by inclining the manometer tube; the amplification factor is $1/\cos \alpha$, where α is the angle between the tube and the vertical. 4. Colored water can be substituted for mercury, *e. g.,* for recording venous pressure, intestinal pressure, etc.

The use of this apparatus is not limited to manometric recordings. Any lever with a broad pointer can be substitued for the manometer, and the light beam will then oscillate at the level of the upper margin of the pointer. All difficulties due to the contact of the pointer with the recording paper are thus eliminated. By removing the lamp from the slide and setting the apparatus in a horizontal position, it can be used for recording deflections of a reflecting galvanometer, eliminating the inconvenient photographic methods.

13506

Demonstration of Rh Antibody in Breast Milk.

ERNEST WITEBSKY, GEORGE W. ANDERSON AND ANNE HEIDE.

From the Buffalo General and the Children's Hospitals, the Departments of Pathology and Bacteriology, Gynecology and Obstetrics, Medical School, University of Buffalo.

Landsteiner and Wiener[1, 2, 3] recently described a new blood factor referred to by them as the Rh factor. Rabbits and guinea pigs when treated with rhesus monkey blood, produced antibodies that

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[1] Landsteiner, K., and Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 223.

[2] Landsteiner, K., and Wiener, A. S., *J. Exp. Med.,* 1941, **74**, 309.

[3] Wiener, A. S., and Peters, H. R., *Ann. Int. Med.,* 1940, **13**, 2306.

agglutinated about 85% of human blood specimens, regardless of their blood group, while 15% of them were not influenced.

Levine and his coworkers, Stetson, Katzin, Burnham, and Vogel,[4-12] observed that in women who gave birth to a child exhibiting erythroblastosis fœtalis, severe transfusion-reactions would occur rather frequently. Over 90% of these women belonged to the Rh negative group, while their children were Rh positive. The Rh factor present in the child and inherited from the father should, according to the conception of Levine, act as an antigen and stimulate antibody-production in the mother. Conversely, such an antibody would then act upon the blood cells of the baby and be the cause of erythroblastosis. The number of cases studied by Levine and his coworkers seems to be large enough to justify the conclusion that isoimmunization in pregnancy might occur, and that the Rh factor plays an important part in the pathogenesis of erythroblastosis.

There are, however, quite a few problems that require clarification. Among them are: First, it is difficult to explain why only a small percentage of Rh^+ babies who have Rh^- mothers develop the disease; second, it is difficult to understand how antigenic substances originating from the child can enter the circulation of the mother and act as an antigen; third, the majority of Rh^- mothers with erythroblastotic children do not reveal any antibody on serological examination. Why should an antibody causing such a severe pathological change in the child not be demonstrable *in vitro?* Fourth, the Rh antibody usually does not produce hemolysis, but only agglutination. Fifth, because of the anticipated absence of the Rh factor in tissue cells, Rh antibodies are supposed to be absorbed exclusively by the blood cells, while antibodies against the A and B substances would be absorbed more or less by all tissue cells. However, the blood-type specific factors, M and N, have, to our knowledge, not been demonstrated as yet in normal tissue cells either. Why do not the blood factors M and N produce isoimmunization in pregnancy

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[4] Levine, P., and Stetson, R., *J. Am. Med. Assn.*, 1939, **113**, 126.

[5] Levine, P., and Katzin, E. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 343.

[6] Levine, P., Katzin, E. M., and Burnham, L., *J. Am. Med. Assn.*, 1941, **116**, 825.

[7] Levine, P., *Am. J. Obst. and Gynec.*, 1941, **42**, 165.

[8] Levine, P., Vogel, P., Katzin, E. M., and Burnham, L., *Science*, 1941, **94**, 371.

[9] Levine, P., Katzin, E. M., and Burnham, L., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 346.

[10] Levine, P., and Polayes, S. H., *Ann. Int. Med.*, 1941, **14**, 1903.

[11] Levine, P., and Katzin, E. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**,

[12] Levine, P., Burnham, L., Katzin, E. M., and Vogel, P., *Am. J. Obst. and Gynec.*, 1941, **42**, 925.

more frequently? Sixth, many children with erythroblastosis are born alive, and severe anemia develops shortly after birth. Provided that antibodies present in the circulation of the mother are responsible for the damage in the child, it is difficult to see why clinical symptoms in those cases become manifest after the child's birth.

We were given an opportunity to study the sixth point mentioned in a typical case of erythroblastosis fœtalis.*

Mrs. J. P.'s first pregnancy resulted in a late abortion, followed by a second pregnancy with a full-term living infant who died during the third day of its life from undiagnosed causes. On October 18, 1941, the patient was delivered of a 6 lb 8 oz living female infant. On the night of admission to the nursery, the child's temperature was elevated, and slight evidence of jaundice was present. Examination of umbilical-cord blood revealed 58% polymorphonuclear cells and 42% lymphocytes with 58 nucleated red blood cells per 100 white blood cells. The infant's jaundice increased in intensity and on the seventh day of its life, the hemoglobin was 9.6 g per 100 cc of blood.

Examination† for the Rh factor revealed Mrs. P. to be Rh^-, and the child and father Rh^+. The mother's serum, in dilutions up to 1:320, agglutinated Rh^+ blood cells, and behaved very similarly to an anti-Rh guinea pig immune serum obtained from Dr. Landsteiner. However, a few human blood specimens appeared to be Rh^+ with the guinea pig immune serum and Rh^- with the patient's serum. This discrepancy points to the fact already described, that the Rh antibodies present in serum of patients with erythroblastotic children might vary in their potency toward different blood specimens. Therefore, the Rh factor is not a sharply identifiable blood factor when examined by different patients' sera.

The child's anemia and icterus increased constantly in severity during the first week of her life. The question arose as to what factor could bring about such a change in the baby's blood picture after the contact between the mother's circulation and that of the baby's had been disrupted at the time of birth. It was considered worthwhile to examine the breast milk for its content in Rh antibodies. The possible importance of breast milk in the pathogenesis of erythroblastosis had been mentioned in older clinical observations—that of colostrum by Hooker,[13] and by Levine and his coworkers.[12] The breast-milk specimen from patient P was centrifuged, and the layer of fat removed with a swab.

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* We are very much indebted to Dr. Robert McDowell and Dr. Richard Downey for having given us the opportunity to study this case.

† Through the courtesy of Drs. Landsteiner, Levine, and Wiener, sera containing anti-Rh antibodies were at our disposal.

[13] Hooker, S. B., *N. Eng. J. Med.*, 1941, **225**, 871.

Several different methods are used for the demonstration of the Rh factor. Landsteiner and Wiener[2] mixed decreasing dilutions of their guinea pig immune serum with the blood cell suspensions. They obtained the best results by examining the sediment formed. Typical differences in positive and negative sediments are illustrated in Landsteiner and Wiener's paper. Levine keeps the mixtures of patient's serum and blood cells under investigation at 37°C for from 30 to 60 minutes and then spins them down in a centrifuge. The observations of the sediments formed gave the most satisfactory results in our experiments when breast milk was examined for anti-Rh antibodies.

The experiment to be recorded was carried out in the following way: About 1% blood-cell suspensions were prepared from nine blood clots all belonging to Group O. One tenth cc of each suspension was then added to: (1) 0.1 cc of a 1:30 dilution of anti-Rh guinea pig serum; (2) 0.1 cc of a 1:20 diluted serum of patient P; (3) 0.15 cc of breast milk of patient P; (4) 0.1 cc of saline solution. The tubes were then kept for about 2 hours in the incubator at 37°C. Rows 1, 3, and 4 in Table I were examined as to the sediment formed. Row 2 was spun down and observed for agglutination. The results obtained are shown in Table I.

As can be seen from Table I, blood specimens found to be Rh^+ with both the guinea pig immune serum and the patient's serum, gave a positive reaction with the milk also, while the Rh^- specimens were not influenced by the milk either. Forty blood specimens all belonging to Group O revealed a perfect parallelism when examined with the patient's serum and with the milk. Blood specimens that reacted positive with the guinea pig serum and negative with the patient's serum were negative with the milk. The specificity of the reaction was ascertained by examining 10 specimens of milk from different

TABLE I.
Determination of the Rh Factor in 9 Blood Specimens Belonging to Group O.

Serum	Blood Specimens								
	1	2	3	4	5	6	7	8	9
1. Guinea-pig serum	—	—	+++	+++	+++	—	+++	+++	++
2. Patient's serum	—	—	++	+++	+++	—	+++	+++	+++
3. Breast milk	—	—	+++	+++	+++	—	+++	+++	+++
4. Saline control	—	—	—	—	—	—	—	—	—

+++ Very strong agglutination or characteristic sediment-formation.
++ Strong but less marked reaction.
— No agglutination or characteristic sedimentation.

mothers. Occasionally, a positive reaction was observed with the control milk specimens. However, these reactions were not common and did not show any correlation with the distribution of the Rh factor. The milk, therefore, seems to contain the same Rh antibody as does the blood serum. Whether Rh antibodies present in the milk are absorbed unaltered from the intestinal canal, and thus, constitute a contributing factor in bringing about the infant's anemia in certain instances, cannot be decided.

It is to be noted that the anti-Rh antibody titer in the milk was very low. Upon dilution, the milk lost its capacity to react with Rh^+ cells. The demonstration of Rh antibodies in the serum of patients with erythroblastotic children succeeds only in a minority of cases. Because of the marked difference in titer between milk and blood serum, it might be even more difficult to find cases of erythroblastosis where it is possible to demonstrate the Rh antibody in the breast milk. No statement can be made regarding the titer of Rh antibodies in colostrum, because we obtained the first milk specimen about a week following delivery.

On the fourteenth day of life, the baby's hemoglobin was down to 7 g per 100 cc of blood, in spite of the fact that 2 blood transfusions with the father's blood had been given. About this time the breast feedings were discontinued. Rh^- blood (but, of course, not the mother's blood) was transfused to the child in consideration of the possible presence of potent Rh antibodies in the baby's circulation. Following the discontinuation of breast feedings and the transfusion of Rh^- blood, the child made a gradual improvement and was discharged from the hospital in an improved condition.[‡]

Conclusion. The occurrence of an Rh antibody in the breast milk of a woman with an erythroblastotic child is described. The patient's serum, too, contained a very potent antibody against Rh^+ blood cells.

[‡] The child's mother belonged to group O, the child and father to Group A. The iso-agglutinin titer of the mother's serum was extremely high; anti-A reached a titer of 1:9640, anti-B, 1:1280. The iso-antibodies, anti-A and anti-B, were present in the breast milk too. The titer of the iso-antibody, anti-A, was 1:100.

13507

Toxicity of *p*-Aminobenzoic Acid.

C. C. Scott and E. B. Robbins. (Introduced by K. K. Chen.)

From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis.

Recent investigations have shown that *p*-aminobenzoic acid is a substance of biological interest. Since this compound possesses a curative action for achromotrichia of rats and mice and is necessary for proper reproduction and lactation in rats, it appears to be a vitamin of the B complex.[1, 2, 3] In addition, the bacteriostatic action of the sulfonamides is inhibited by *p*-aminobenzoic acid.[4] Further, it probably is important for metabolism of plants in general.[5] In human subjects, darkening of gray hair has resulted from ingestion of this substance.[6] Because of these studies, the importance of toxicity determinations of *p*-aminobenzoic acid becomes evident.

Acute Toxicity. Mice, rats, and dogs were employed. For oral studies, the free acid was administered by stomach tube. The sodium salt was prepared for the intravenous toxicities since the free acid is too insoluble. *p*-Aminobenzoic acid is more toxic to dogs and mice than to rats. The median lethal dose (LD_{50} ± standard error) in mice was observed to be 2.85 ± 0.4, that in rats greater than 6, and that in dogs between 1 and 3 g per kg. It is interesting that the sodium salt of *p*-aminobenzoic acid, in contrast with the free acid, is more toxic to rats than mice by intravenous injection. No explanation is as yet available for this reversal of toxicity. The results are shown in Table I.

Orally, toxic signs in mice consisted only of weakness and loss of normal posture, death occurring in several hours. Mild clonic convulsions with death in 5-10 minutes resulted from intravenous lethal doses in mice and rats.

In dogs, 1-2 days passed before death occurred. Toxic signs were tremors, listlessness, and weakness; 2 animals vomited; and tonic and clonic convulsions were observed in the dog which received the largest dose. Autopsies on the animals which received lethal doses showed acute gastro-enteritis with hemorrhages apparently of cap-

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[1] Ansbacher, S., *Science,* 1941, **93**, 64.

[2] Martin, G. J., and Ansbacher, S., *J. Biol. Chem.,* 1941, **138**, 441.

[3] Sure, B., *Science,* 1941, **94**, 167.

[4] Woods, D. D., and Fildes, P., *J. Soc. Chem. Ind.,* 1940, **59**, 133.

[5] Wiedling, S., Science, 1941, **94**, 389.

[6] Sieve, B. F., *Science,* 1941, **94**, 257.

TABLE I.
Acute Toxicity of *p*-Aminobenzoic Acid.

Substance	Animal	Administration	Dose, g per kg	No. died / No. used	LD_{50} ± standard error g per kg
Free Acid	Mouse	Oral	2.5	1/5	2.85 ± 0.4
			3.0	5/8	
			3.5	9/10	
			4.0	8/8	
	Rat		2.0	0/8	>6.0
			2.5	0/7	
			3.0	0/7	
			3.6	0/8	
			4.2	0/8	
			5.0	0/8	
			6.0	0/8	
	Dog		0.5	0/3	between 1.0 and 3.0
			1.0	1/3	
			1.5	1/1	
			2.0	1/1	
			3.0	1/1	
Sodium Salt	Mouse	Intravenous	3.0	1/13	4.60 ± 0.21
			4.0	1/13	
			5.0	11/18	
			6.2	8/8	
	Rat		1.6	0/5	2.76 ± 0.24
			2.0	0/5	
			2.4	2/5	
			3.2	3/3	
			4.0	4/5	

illary origin in the small intestine. Acute necrosis of the liver occurred in the dogs given the two highest dosages.

Feeding Experiments. By stomach tube, 6 rats received 0.6, and 7 rats 1.4 g per kg of *p*-aminobenzoic acid daily for 28 days. As controls, 7 rats were given 1.5 cc of water by stomach tube for the same period. All the animals were fed a standard stock diet. The rats were 6 weeks old when medication was started. Throughout the experimental period, the gain in weight for all 3 groups was the same. Autopsies on all of these animals were entirely negative. It is evident from these chronic studies, as well as the acute toxicities, that the rat is very resistant to *p*-aminobenzoic acid.

Summary. 1. In mice, rats, and dogs, the acute toxicity of *p*-aminobenzoic acid by mouth has been determined. The drug is more toxic to mice and dogs than to rats. By intravenous injection the sodium salt of *p*-aminobenzoic acid is more toxic to rats than to mice. 2. Oral doses of *p*-aminobenzoic acid greater than 1.0 g per kg to dogs may cause death accompanied by acute gastro-enteritis and hemorrhages into the small intestine. Acute necrosis of the liver may result from doses of 2.0 g per kg or larger. 3. Rats tolerate daily doses of 1.4 g per kg by mouth for about a month without inhibition of growth or pathological changes.

13508

Lactobacillus casei ε Factor in the Nutrition of the Chick.*

R. C. MILLS, G. M. BRIGGS, JR., C. A. ELVEHJEM AND E. B. HART.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wisconsin.

The activity of a norit eluate fraction required by *Lactobacillus casei* ε, in promoting growth in chicks receiving purified rations, was recently reported from this laboratory.[1] Further studies have been made on the rôle of this fraction in chick nutrition and the results are presented here. It has been found that the *Lactobacillus casei* ε eluate fraction contains a factor or factors essential for growth, hemoglobin formation, and normal feathering in growing chicks.

The basal ration used had the following percentage composition: dextrin 59, purified casein 18, cartilage 10, kidney residue 2, salts IV[2] 5, $CaHPO_4$ 1, and soybean oil 5. Each kg of ration also contained thiamin 3 mg, riboflavin 4 mg, pyridoxine 4 mg, Ca pantothenate 15 mg, nicotinic acid 100 mg, choline 1.5 g, and inositol 1 g. Each chick received 2 drops of haliver oil per week. In more recent work 10% gelatin and 0.2% cystine were substituted for cartilage in order to produce a more deficient ration. It has been found that gelatin and cystine can replace cartilage in promoting growth.[3]

Day-old White Leghorn chicks were obtained either from a commercial hatchery or from the University poultry department. They

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. We are indebted to Merck and Company, Rahway, N.J., for the synthetic vitamins; to Abbott Laboratories, North Chicago, Ill., for haliver oil; to Wilson Laboratories, Chicago, Ill., for cartilage and solubilized liver extract; and to Allied Mills, Peoria, Ill., for soybean oil.

[1] Hutchings, B. L., Bohonos, N., Hegsted, D. M., Elvehjem, C. A., and Peterson, W. H., *J. Biol. Chem.*, 1941, **140**, 681.

[2] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 458.

[3] Unpublished data.

were divided into groups of 6 and placed in electrically heated brooders with raised screen bottoms. Water and the experimental ration were supplied *ad libitum*.

The kidney residue used in these rations is prepared by extracting dried defatted kidney with large volumes of hot water and 50% alcohol with additional refluxing with 50% methanol. It is used as a source of biotin.

Hemoglobin was determined by the method adapted for the Evelyn photoelectric colorimeter, with the following slight modification: The samples were centrifuged at high speed for 1 minute after addition of the alkali in order to clarify the solutions and readings were made immediately.

The results are summarized in Table I. Chicks on the basal ration grew very poorly, averaging 124 g at 4 weeks of age. Addition of solubilized liver extract at a level of 2% of the ration supplied all other essential factors for the chick, since optimum growth was obtained. A norit eluate prepared from solubilized liver extract by the method of Hutchings, *et al.*,[4] also gave marked growth responses, but the maximum weight was about 40 g below that obtained with the solubilized liver extract. The difference is still more marked when a biotin concentrate is substituted for kidney residue. This difference indicates the existence of at least one other unidentified factor in the solubilized liver extract.

Very poor feathering was also noted on chicks receiving the basal ration. The poor condition of the feathers first becomes noticeable at 3 weeks. After this period, feather formation is very slow or absent, and more and more feathers break off until few large feathers remain. Fig. 1 shows a chick maintained on the basal ration for 5 weeks. At this time the feathers are rough, shaggy, sparse and brittle; many are broken off. The bird in Fig. 2 is the same age, but received 2% solubilized liver extract in the ration. The feathering is normal

TABLE I.
Effect of Supplements on Growth and Anemia.

Supplement to basal ration	No. of chicks	Avg wt 3 wks g	Avg wt 4 wks g	Hemoglobin at 4 wks g/100 cc	Feathering
None	30	105	124	6.13	Poor
Norit eluate equivalent to 5% solubilized liver extract	18	161	189	8.09	Good
2% solubilized liver extract	60	172	232	8.40	”
2% glycine + 1/2% arginine	6	107	120		Poor

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[4] Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

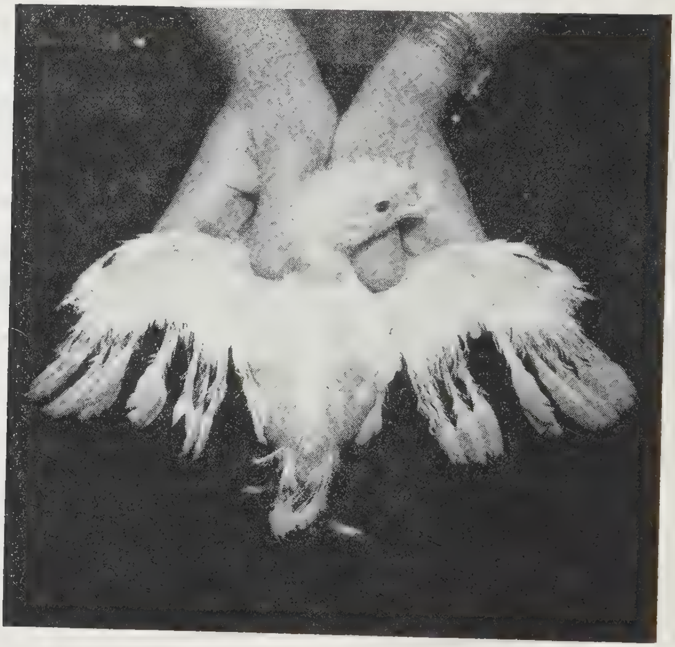

FIG. 1.
Chick on basal ration. Age 5 weeks. Note poor feathering.

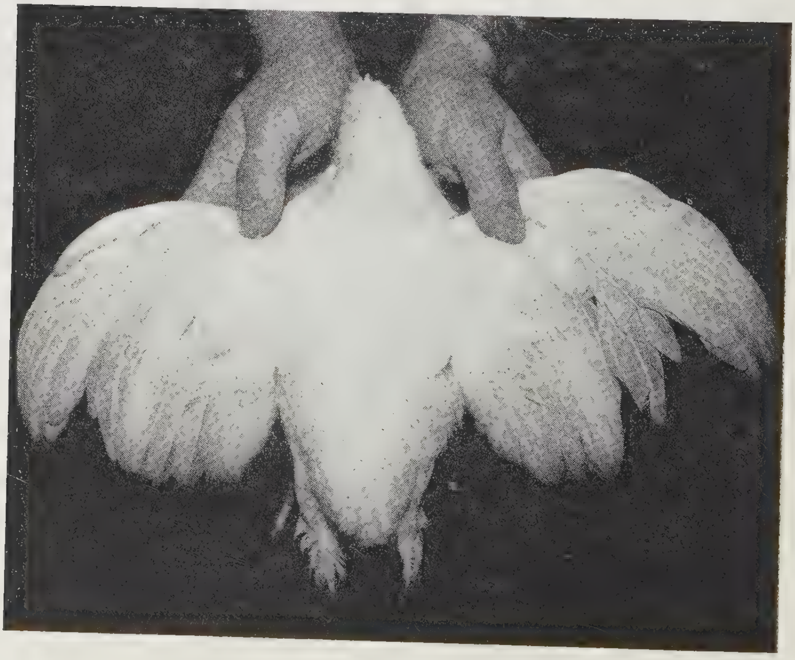

FIG. 2.
Chick on basal ration plus 2% solubilized liver extract. Age 5 weeks.

and complete. The norit eluate also produces normal feathering.

The poor feathering on this basal ration is distinct from that ascribed to a glycine and arginine deficiency;[5] the typical lacy appearance of the feathers on the amino acid deficiency is absent here. A group of chicks was given arginine and glycine at adequate levels. No improvement in feathering above the basal was noted, and there was no increase in growth.

Another symptom noted on the basal ration at 4 weeks is a reduced hemoglobin content of the blood. The average hemoglobin content of the blood of chicks on the basal ration was 6.13 g per 100 cc with some values as low as 3 g. The chicks receiving solubilized liver extract had an average level of 8.40 g, and those getting the norit eluate, 8.09 g. The anemia becomes more severe as the chicks grow older.

The properties of the *Lactobacillus casei* ϵ eluate fraction are similar to those reported for several other chick factors. Factor U,[6] the alcohol precipitate factor,[7] Hogan's antianemia factor,[8] and the norit eluate fraction are all alcohol insoluble and are all adsorbed on Fuller's earth at an acid pH. The identity of "folic acid" and the norit eluate factor has already been discussed.[4] The occurrence of anemia on our basal ration and its prevention by the norit eluate is suggestive of the identity of the norit eluate fraction with Hogan's antianemia factor.

Summary. Further studies on the rôle of the *Lactobacillus casei* ϵ eluate factor in chick nutrition are reported. Purified preparations of the factor promote growth, hemoglobin formation, and normal feathering.

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[5] Hegsted, D. M., Briggs, G. M., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **140**, 191.

[6] Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.*, 1938, **125**, 687.

[7] Schumacher, A. E., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, 1940, **135**, 313.

[8] Hogan, A. G., and Parrott, E. M., *J. Biol. Chem.*, 1940, **132**, 507.

13509

Effect of Estrone* on Lactogen Content in Pituitary and Blood of Male Rabbits.†‡

Joseph Meites and C. W. Turner.

From the Department of Dairy Husbandry, University of Missouri, Columbia, Mo.

Reece and Turner[1, 2] were the first to demonstrate that the administration of estrone or estradiol will increase the lactogenic hormone in the pituitaries of male and female rats, and male guinea pigs. Later, testosterone propionate[3] and diethylstilbestrol[4, 5] were likewise shown capable of augmenting the lactogenic potency of pituitaries in female rats and male guinea pigs. The present authors have obtained a maximum rise with estrone and diethylstilbestrol of 410 and 438% respectively in the lactogen content of the pituitaries of immature male guinea pigs. In actual units of lactogenic hormone, the immature male guinea pigs (weighing an average of 334 g), contains 3.06 Reece-Turner units (R.-T.U.) per pituitary. Upon the administration of estrone or diethylstilbestrol the average lactogen content per pituitary rose to a maximum of 17.75 and 16.50 R.-T.U. respectively.[5] This seems highly significant in view of the fact that the lactating guinea pig pituitary has been observed at no time during the lactation cycle to contain more than 18.38 R.-T.U.[6]

In addition to the increase in the lactogenic hormone of the pituitary which can be obtained with estrogen, lactation itself has been initiated by the administration of estrogens.[2-4, 7-9] This seems to

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* The estrone used in this experiment was kindly furnished by Dr. H. C. Spruth of the Abbott Laboratories.

† Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 803.

‡ This study has been aided in part by a grant from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

[1] Reece, R. P., and Turner, C. W., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 402.

[2] Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Bul.* 266, 1937.

[3] Reece, R. P., and Mixner, J. P., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 66.

[4] Lewis, A. A., and Turner, C. W., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 439.

[5] Meites, J., and Turner, C. W., unpublished data.

[6] Holst, S., and Turner, C. W., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 479.

[7] Folley, S. J., Scott Watson, H. M., and Bottomley, A. C., *J. Physiol.*, 1940, **98**, 15.

[8] Lewis, A. A., and Turner, C. W., *Proc. Am. Soc. An. Prod.*, 1940, **33**, 63.

[9] Walker, S. M., and Stanley, A. J., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 50.

indicate that estrogen causes the pituitary to release more lactogen into the blood stream. However, there has been some doubt expressed as to whether the increase in the lactogen content of the pituitary which follows upon estrogen treatment actually represents an increased release of lactogen into the blood stream or only a storage of lactogen in the pituitary.

Recently, it has been reported that lactogen can be detected in the blood stream of normal, pregnant and lactating rabbits.[10] As little as 1 cc of whole untreated rabbit blood was found to give positive responses when injected intracutaneously over the crop sacs of common pigeons. It was of interest, therefore, to determine whether we could demonstrate a simultaneous increase of lactogen in the blood stream as well as in the pituitary of normal male rabbits upon the injection of estrone.

Methods. Male New Zealand white rabbits, weighing approximately 2500 g, were used in this experiment. Ten rabbits served as a control group and received no estrone, while 3 experimental groups were injected subcutaneously with 3 different levels of estrone. Approximately 10 cc of venous blood (from an ear vein) was obtained from each rabbit both before the injections of estrone were begun and after the last injection. Potassium oxalate was used to keep the blood from clotting. All the experimental rabbits were injected with estrone for a 10-day period and killed on the eleventh day.

The whole untreated blood from each rabbit group was assayed by injecting a total of 0.50 and 1.00 cc intradermally over the two crop glands of 10 pigeons for 4 days and then determining the number of positive responses in the crop glands on the fifth day. The pituitaries of each group were macerated separately in a small agate mortar, taken up in a little distilled water, and assayed by the Reece-Turner pigeon method.[2]

Results. All 3 dosage levels of estrone brought a considerable increase in the lactogen content of the male rabbit pituitary (Table I). It will be noted that 5000 I.U. estrone elicited a significantly smaller increase in lactogenic hormone than was secured with 500 and 1000 I.U. It was also observed that the mammary glands of the rabbits receiving the highest dosage of estrone were less extensively developed than in the animals receiving the smaller amounts of estrone. Similarly, it has been observed[5] that large dosages of diethylstilbestrol will produce less increase in the lactogen content of the pituitaries of male guinea pigs than can be obtained with

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[10] Meites, J., and Turner, C. W., *J. Clin. Endocrinol.*, 1941, **1**, 918.

TABLE I.

Effect of Estrone on Lactogen Content of the Pituitary.

No. of rabbits per group	Avg body wt, g	Amt of estrone inj. per animal		Pituitary wt, mg	Avg Reece-Turner lactogen units,* pituitary	Avg R.-T. units, mg pit. tissue	Avg R.-T. units, 1000 g body wt	Avg % inc. in lactogen, 1000 g body wt
		Daily I.U.	Total I.U.					
10	2545	Controls		26.43	2.24	.084	.88	
7	2761	50	500	24.37	8.25	.340	3.00	240
7	2589	100	1000	32.00	12.16	.380	4.69	433
9	2598	500	5000	29.10	6.06	.208	2.29	160

*One Reece-Turner lactogen unit = .045 International Units (Meites, J., Bergman, A. J., and Turner, C. W., *Endocrinol.*, 1941, **28**, 707.

smaller doses. However, even the highest amount of diethylstilbestrol used (100.0 mg) was found to produce some increase. In comparison to the high lactogen content of the pituitary which can be secured with estrogens in the immature male guinea pig, it is interesting to observe that the 3 levels of estrone injected failed to increase the lactogen content of the pituitaries of the experimental rabbits to levels at all comparable to that of the lactating rabbit (60.00 R.-T.U. at 5 days postpartum[5]). However, it is possible that dosages of estrone other than those used in this experiment might have increased the lactogenic potency of the male rabbit pituitary to a greater degree than was observed here.

The data in Table II reveal that a very definite increase was secured in the lactogen content of the blood stream following the administration of estrone. These results prove that the measured increase in the lactogen content of the pituitary which can be secured upon the administration of estrone occurs simultaneously with an increase in the release of lactogen into the blood stream. This explains why

TABLE II.

Effect of Estrone on Lactogen Content in Blood.

Total amt estrone inj. per rabbit, I.U.	Amt rabbit blood inj. per pigeon, ml	No. + responses in 10 pigeons before estrone admin.	No. + responses in 10 pigeons after estrone admin.
Controls	1.00	4	
	0.50	2	
500	1.00	5	10
	0.50	2	10
1000	1.00	4	9
	0.50	4	7
5000	1.00	6	8
	0.50	4	5

lactation can be initiated in some animals upon the administration of proper amounts of estrogens.

Summary. Three groups of male rabbits were injected with 3 different levels of estrone in order to determine whether estrogen could effect a simultaneous rise in the lactogenic hormone of the pituitary and blood stream. Assays of the pituitaries revealed that a considerable increase in lactogen was obtained, although the highest dosage of estrone (5000 I.U.) gave a smaller increase in lactogen than was secured with the two lower levels (500 and 1000 I.U.). Assays of the whole, untreated rabbit blood also showed that a definite increase in the release of lactogen into the blood stream was secured following the injection of estrone. These findings are believed to account for the initiation of lactation following suitable estrogen treatment.

13510

Lactogenic Content of Pituitaries of Pseudopregnant Rabbits.*†

JOSEPH MEITES AND C. W. TURNER.

From the Department of Dairy Husbandry, University of Missouri, Columbia, Mo.

It has been suggested that copious lactation can be initiated only when (a) there is a well developed mammary gland present and (b) when there is a high lactogen content in the anterior pituitary.[1] After the middle of pregnancy one of these two factors is present, namely, a well developed mammary gland, but the production of lactogenic hormone by the pituitary remains at too low a level to initiate abundant lactation. Thus in the rat,[2, 3] guinea pig,[4] and rabbit,[1, 4] the lactogen content of the pituitary remains as low during pregnancy as in the non-pregnant state. Almost immediately following parturition, however, there is a great increase in the lactogenic hormone of the pituitary (from 200 to over 400%), and full lactation is initiated.[2, 3, 4] An increased lactogen content in the

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* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 802.

† This study has been aided in part by a grant from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

[1] Turner, C. W., and Meites, J., *Endocrinol.*, 1941, **29**, 165.

[2] Reece, R. P., and Turner, C. W., *Missouri Agr. Exp. Sta. Bull.* 266, 1937.

[3] Reece, R. P., Hathaway, I. R., and Davis, H. P., *Dairy Science*, 1939, **22**, 1.

[4] Holst, S., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 479.

urine has also been demonstrated in postpartum women.[5]

Inasmuch as the mammary glands of the rabbit become well developed during pseudopregnancy, it was of interest to determine whether the content of lactogenic hormone in the pituitary at the end of pseudopregnancy would account for the absence of any marked degree of milk secretion in such animals. Seven normal, mature New Zealand white rabbits served as control animals, while 11 similar rabbits were each injected intravenously with 100 I.U. chorionic gonadotropin (Korotrin)[‡] in order to induce pseudopregnancy. Twenty days later both the control and pseudopregnant rabbits were killed, and their pituitaries were removed for assay. In 6 of the 11 pseudopregnant rabbits it was found possible to express either a slight amount of milk or serous secretion from the teats. The pituitaries of each group were assayed quantitatively for lactogen by the Reece-Turner pigeon method.[2]

The assays revealed that the normal mature female rabbits which served as controls contained an average of 10.05 Reece-Turner (R.-T.) lactogen units per pituitary, while the pseudopregnant rabbits contained an average of 10.25 R.-T. units per pituitary. Previously it had been found that rabbits on the twentieth day of pregnancy contained an average of 9.75 R.-T. units of lactogen per pituitary.[1] Thus the normal mature rabbit, the pseudopregnant rabbit and pregnant rabbit all contain approximately the same amount of lactogenic hormone in their pituitaries. In contrast, the pituitaries of lactating rabbits 5 days postpartum contain an average of 60.0[6] R.-T. units per pituitary. It is concluded, therefore, that the reason no substantial lactation occurs in the rabbit during pseudopregnancy, as also during pregnancy, is because the amount of lactogenic hormone in the pituitary is too low. This receives support from the fact that pseudopregnant rabbits can easily be brought into full lactation upon the administration of lactogenic hormone.[7, 8, 9]

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[5] Meites, J., and Turner, C. W., *J. Clin. Endocrinol.*, 1941, **1**, 918.

[‡] We wish to express our thanks to the Winthrop Chemical Company for making this hormone available to us.

[6] Meites, J., and Turner, C. W., unpublished data.

[7] Gardner, W. U., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 196, 1933.

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13511 P

Induced Ovulation in Amphiuma.

ADRIAN KAMMERAAD. (Introduced by R. Ashman.)

From the Department of Anatomy, School of Medicine, Louisiana State University, New Orleans.

Since the work of Wolf[1] and Houssay, Giusti and Lascano-Gonzalez,[2] ovulation has been induced in about 21 species of amphibia (Rugh).[3] Induced ovulation in *Amphiuma tridactylum* Cuvier can now be added to this list.

Very little is known about the life history of this amphibian. Baker[4] attempted to induce ovulation by means of injections of frog and catfish pituitary glands. Since only one female Amphiuma was used and the results were negative, the work obviously needed to be repeated. The author is greatly indebted to Dr. F. H. Wilson of Tulane University for supplying the animals used in this experiment and for information concerning their life history and habits.

Materials and Methods. Five adult female specimens of Amphiuma with palpable eggs in their ovaries were collected in June and injected daily with fresh cattle or frog pituitary glands macerated in distilled water or an aqueous suspension of desiccated sheep pituitary powder. Intraperitoneal, intramuscular and subcutaneous routes were used. The animals were kept in covered boxes, the bottoms of which were covered with 3 inches of dirt from the normal habitat.

Observations. Beginning June 13, 1941, animal No. 1 was injected daily with macerated male and female frog pituitary glands. On June 23 she laid one egg and another on June 24. A total of 9 female and 13 male frog pituitaries using all three routes were required to produce ovulation. Injections were continued, using a total of 2 cc of aqueous extract of cattle pituitary (1 cc = .2 of a gland). On June 28 the animal died and at autopsy no ovulated eggs could be found in the abdominal cavity.

Animal No. 2 was injected intraperitoneally with cattle pituitary extract beginning June 13, 1941. A total of 18 cc (1 cc = .2 of a gland) was given in daily doses of 2-4 cc. The animal died June 19. No eggs were laid but at autopsy several eggs (6-10) were found free in the abdominal cavity. Some of the eggs had burst and an accurate count was impossible.

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[1] Wolf, O. M., *Anat. Rec.*, 1929, **44**, 206; PROC. SOC. EXP. BIOL. AND MED., 1929, **26**, 692.

[2] Houssay, B. A., Giusti, L., and Lascano-Gonzalez, J. M., *Rev. de la Soc. Argentina de Biol.*, 1929, **5**, 397.

[3] Rugh, R., *Biol. Bull.*, 1934, **66**, 22; *J. Exp. Zool.*, 1935, **71**, 149.

[4] Baker, L. C., *J. Tenn. Acad. Sci.*, 1937, **12**, 206.

Animal No. 3 was injected daily with 5 cc of an aqueous suspension of desiccated sheep pituitary powder beginning June 13. The intraperitoneal route was used. The animal died June 18. No eggs were laid and at autopsy no ovulated eggs could be found in the abdominal cavity.

Animal No. 4 was given daily subcutaneous injections of 2 cc of cattle pituitary extract beginning June 20. The animal died over the week-end of June 28 after receiving a total of 12 cc (1 cc = .2 of a gland). No eggs were laid and at autopsy no ovulated eggs could be found in the abdominal cavity.

Animal No. 5 was injected intramuscularly with cattle pituitary extract beginning June 20. On June 24 after receiving 4 cc of the extract (1 cc = .2 of a gland) this animal was found in the process of laying eggs. A total of 35 eggs was laid that day. Nine more were found on June 25 and 5 more on June 27.

Animals No. 6 and No. 7 were untreated. Neither one laid any eggs during the period of observation. Amphiuma has never before been known to lay eggs in captivity.

Discussion. Since our knowledge of the mating habits and life history of Amphiuma is fragmentary, it is impossible to draw any extensive conclusions from this work. Further work on this problem is in progress. According to Baker[4] eggs are normally laid in August. Observations on this point are few but ovulation induced in June undoubtedly represents a precocious process.

It is suggested that the intramuscular and subcutaneous routes are more efficacious because at autopsy those animals which were injected intraperitoneally, contained large amounts of fluid in the abdominal cavity. This fluid resembled the injected material which in all probability failed to be absorbed by the peritoneum.

Conclusion. Ovulation in Amphiuma can be induced prior to the normal egg-laying season by injections of cattle and frog pituitary suspension and extract.

13512

Action of Certain Germicides on Meningococcus.

C. Phillip Miller. (With the technical assistance of Doretta Schad.)

From the Department of Medicine and the A. B. Kuppenheimer Research Foundation, University of Chicago.

Susceptibility to death by chemical poisoning is a significant aspect of the biology of any bacterium and especially of a pathogen as important as meningococcus. This microörganism is generally supposed to be a delicate one, easily killed by antiseptic agents, but it is rarely included among the gram-negative organisms employed in the estimation of the germicidal activity of disinfectants, probably because it is somewhat exacting in its requirements for abundant growth on artificial media.

Methods. Most of the experiments were performed with a Type I strain of maximal virulence for mice, *i. e.,* less than 10 organisms sufficed to initiate a fatal infection by the mucin method.[1] In some experiments Type II organisms were also used, but in no experiment in which a comparison was made was any significant difference detected in the susceptibility of the two types to the germicides tested.

The meningococci were grown on the surface of a solid medium consisting of tryptic digest of egg-white, one percent glucose, buffer and agar,[2] in ordinary pint medicine bottles.

After 18 hours' growth the microörganisms were recovered in saline, centrifuged and resuspended in various dilutions of the chemical agent to be tested. Usually 10 cc volumes were used containing 5 to 10 billion microörganisms per cc. Care was taken to insure thorough mixing and dispersion of the bacteria. The suspensions were allowed to stand at room temperature for 15 to 30 minutes and then centrifuged. The sediment, carefully drained of supernatant, was washed by resuspending in 10 cc of saline and centrifuging again. A loopful of the washed sediment was then streaked onto the surface of a freshly poured blood agar plate, which was incubated and examined twice a day for 3 days because occasionally some colonies grew out slowly. Colonies of doubtful appearance were checked by microscopic examination of stained smears and subcultures.

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[1] Miller, C. P., and Castles, Ruth, *J. Inf. Dis.*, 1936, **58**, 263.

[2] Miller, C. P., and Castles, Ruth, Proc. Soc. Exp. Biol. and Med., 1930, **28**, 123.

Unless this routine was followed, contradictory findings resulted. If, for instance, the bacterial growth was washed off the media with the germicide itself, the lethal action of the latter was much reduced by its contact with the agar and protein degradation products comprising the media, and even if the culture was washed off with saline and added directly to the germicide, enough ingredients of the media were carried along to diminish appreciably the effectiveness of the antiseptic.

Results and Comments. The tables show only the results of the cultures at and near the limit of each chemical's ability to kill all of the test organisms under the conditions described.

TABLE I.
Viability of Meningococci After Treatment with Germicides.

Chemical	Concentration		Growth
Salts of heavy metals			
Mercuric Chloride	.025%	.00092 *M*	0
	.012	.00046	0 or very few‡
	.006	.00023	++
Phenyl Mercuric Nitrate	.006%	.000184 *M*	0
	.003	.000092	0 or +‡
	.0015	.000046	0 or +‡
	.00075	.000023	++
Copper Sulphate	.012%	.000751 *M*	0
	.006	.000376	0 or very few‡
	.003	.000188	+
	.0015	.000094	++
Halogens			
Chlorine Water	1/4 saturated		0
	1/8		+
	1/16		++
Compound Solution Iodine, U.S.P. Lugol's Solution	2.5%		0
	0.62		+
	0.16		++
Sodium Fluoride	5.0%	1.19 *M*	+
	2.5	.595	+±
	1.0	.238	++
Sodium Azide, NaN_3	10.0%	1.535 *M*	0 to +‡
	5.0	.768	+±
	1.0	.1535	++
Potassium Tellurite	.2%	.00788	0
	.04	.001576	+
	.008	.000315	++
Organic Compounds			
Phenol	1.0%	.015 *M*	0
	0.25	.026	++
	0.06	.0063	++

Chemical	Concentration		Growth
Formaldehyde	0.4%	.132 *M*	0
	0.1	.033	0
	0.025	.008	+
Propylene Glycol	60.0%	7.88 *M*	0
	50.0	6.56	0 or +‡
	40.0	5.26	0 or +‡
	30.0	3.94	++
Alcohol	30.0%	6.51 *M*	0
	20.0	4.33	0 or ±‡
	10.0	2.16	+
*Quinone	.01%	.000925 *M*	0
	.001	.0000925	++
	.0001	.00000925	++
*Quinhydrone	.01%	.000458 *M*	0
	.001	.0000458	++
	.0001	.00000458	++
*Iodoacetic Acid	.1%	.00537 *M*	0
	.01	.000537	++
	.001	.0000537	++
Synthetic Detergents: anionic			
†Duponol L S			
Sodium Oleyl Sulphate	.05%		0
	.01		++
	.002		++
†Triton W-30			
Sodium Salt of Alkyl Phenoxy Ethyl Sulphonate	.05%		0
	.01		+
	.002		++
Synthetic Detergents: cationic			
†Hydrocide			
Alkyl Hydroxy Benzyl Dimethyl Ammonium Phosphate	.25%		0
	.05		++
	.01		++
†Zephiran			
Alkyl Dimethyl Benzyl Ammonium Chloride	.025%		0
	.005		0 or +‡
	.001		++

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*These compounds were very kindly supplied by Dr. E. S. G. Barron of this Department.

†These compounds were very kindly supplied by Dr. Zelma Baker of this Department.

‡Designates concentrations of germicide at which the results were variable: *i.e.*, growth in some experiments, no growth in others.

Compounds containing a heavy metal were the most effective germicides and of these phenyl mercuric nitrate was the best. This is in accord with the findings of Weed and Ecker,[3] who found it the most potent of a number of mercurials studied. Next in order were the two inhibitors of respiratory enzymes: quinone and quinhydrone, which were lethal in concentrations of 0.01% (= 0.0009 and 0.00045 *M* respectively). Iodoacetic acid was somewhat less active. These results were to be expected from the observations of Barron.[4, 5]

The next most active germicide was the cationic detergent Zephiran, which killed regularly at 0.025% and usually at 0.005%. The other cationic detergent, Hydrocide, was much less effective, failing to kill at 0.05%. The two anionic detergents, Duponol and Triton W.30, were more effective than Hydrocide and less active than Zephiran. The activity of the anionic compounds is surprising in view of the work of Baker, Harrison and Miller,[6, 7, 8] who studied the effect of a number of detergents on the metabolism of 6 gram-negative organisms and on the viability of three, and found the gram-negatives resistant to the anionic compounds. Similar observations are reported by Dubos.[9]

Among the common antiseptics, alcohol was more active than was to be expected, killing most of the test organisms at 20% concentration. Formaldehyde was regularly lethal in concentrations of 0.1%. But phenol, chlorine water and Lugol's solution (Compound Solution of Iodine, U.S.P.) required much higher concentrations than had been anticipated. Phenol was ineffective in less than one percent concentration, chlorine water in less than one-fourth saturation and Lugol's solution in less than 2.5%. The latter was found by Nye[10] to be the most effective of the germicides tested with *Staphylococcus aureus.*

The action of propylene glycol on meningococcus was similar to that found by Robertson[11] on pneumococcus.

Potassium tellurite was lethal at a concentration of 0.2% but not at 0.04%. This was first used by Conradi and Troch[12] and later by Fleming,[13] who found most gram-negative organisms including gonococcus sensitive to it, although meningococcus seems not to have been tested in his experiments. Rose and Georgi[14] found potassium tellurite in 0.03% concentration to inhibit the growth of gram-negatives.

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[3] Weed, L. A., and Ecker, E. E., *J. Inf. Dis.*, 1933, **52**, 354.

[4] Barron, E. S. G., *J. Biol. Chem.*, 1936, **113**, 695.

[5] Barron, E. S. G., and Friedemann, T. E., *J. Biol. Chem.*, 1941, **137**, 593.

[6] Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **73**, 249.

[7] Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **74**, 611.

[8] Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **74**, 621.

[9] Dubos, Rene J., University of Pennsylvania Bicentennial Conference, 1941.

[10] Nye, Robert N., *J. A. M. A.*, 1937, **108**, 280.

[11] Robertson, O. H., personal communication to the author.

[12] Conradi, H., and Troch, P., *Munch. Med. Woch.*, 1912, **59**, 1652.

[13] Fleming, Alexander, *J. Path. and Bact.*, 1932, **35**, 831.

Quite unexpected were the results with sodium fluoride and sodium azide, which failed to kill meningococci under the conditions of these experiments in concentrations of 5%. The former is a well known inhibitor of glucose fermentation by cells and cell-free extracts. Snyder and Lichstein[15] report that 0.01% sodium azide was sufficient to restrain the growth of gram-negative bacteria on blood agar and so have Mallman, Botwright and Churchill.[16] A second paper by Lichstein and Snyder[17] recommends the addition of sodium azide to media as the most satisfactory method of preventing the spread of Proteus and of exerting a general bacteriostatic action against all gram-negative organisms.

It seems clear from the experiments herein described that meningococcus is unlike other gram-negative bacteria in being resistant to certain chemicals which are toxic to the members of this group usually subjected to germicidal tests. Smith[18] suggested that the Neisseriae might be exceptional among the gram-negatives in their susceptibility to the bacteriostatic action of various dyes, but in most of his experiments he used only *N. catarrhalis* as representative of the whole genus because of the difficulty of growing meningococcus and gonococcus on his standard media.

Summary. Meningococci were subjected to the action of a number of germicides by suspending aliquot quantities in various concentrations of each chemical, removing them by centrifugation, washing and culturing the washed sediment on blood agar plates. Several of the chemicals usually regarded as potent germicides failed to kill all or most of the meningococci.

Conclusions. Meningococci are more resistant to the lethal action of certain chemicals than the gram-negative organisms usually employed in germicidal tests.

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[14] Rose, Kenneth D., and Georgi, Carl E., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 344.

[15] Snyder, M. L., and Lichstein, Herman C., *J. Inf. Dis.*, 1940, **67**, 113.

[16] Mallman, W. L., Botwright, W. E., and Churchill, Elbert S., *J. Inf. Dis.*, 1941, **69**, 215.

[17] Lichstein, H. C., and Snyder, M. L., *J. Bact.*, 1941, **42**, 653.

[18] Smith, Homer W., *Am. J. Hyg.*, 1922, **21**, 607.

13513 P

Changes of Vitamin A Distribution in Choline Deficiency.

Hans Popper* and Herman Chinn.† (Introduced by C. J. Farmer.)

From the Cook County Hospital, the Cook County Graduate School of Medicine, and the Departments of Chemistry and Ophthalmology, Northwestern University Medical School, Chicago.

Choline-deficient diets produce fatty livers (Best[1]). Griffith[2] demonstrated that in addition to the liver changes, young rats develop an acute syndrome with hemorrhagic renal degeneration, splenic enlargement, ocular hemorrhage and thymic involution. Livers of experimental animals made fatty by diets[3] or toxic agents[4] ordinarily are rich in vitamin A which can be seen histologically in the fat droplets.[5] Choline feeding stimulates the removal of vitamin A as well as fat from the liver.[3] It seemed important, therefore, to determine whether a choline deficiency reveals a similar dependence of the vitamin A storage upon fat deposition in the liver. Since human fatty livers may be either rich or poor in vitamin A,[6] this question assumes clinical significance.

Thirty-two rats, 21 to 28 days old, were fed a low choline, low methionine, high cystine diet,[7] supplemented with carotene and the crystalline vitamins.‡ Control animals received 25 mg choline chloride daily. Livers, kidneys and adrenals were examined by routine histologic procedures and for vitamin A under the fluorescence microscope.[6] The vitamin A of some livers was determined chemically.§

Fourteen rats on the deficient diet showed fatty livers of varying

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* Research Fellow of the Cook County Graduate School of Medicine.

† Fellow of the Snyder Ophthalmic Foundation.

Aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Best, C. H., Hershey, J. M., and Huntsman, M. E., *Am. J. Physiol.*, 1932, **101**, 7.

[2] Griffith, W. H., *J. Nutrition*, 1941, **22**, 239.

[3] Thorbjarnarson, T., and Drummond, J. C., *Biochem. J.*, 1938, **32**, 5.

[4] Lasch, F., *Klin. Wchnschr.*, 1935, **14**, 1070.

[5] Popper, H., and Steigmann, F., in preparation.

[6] Popper, H., *Arch. Path.*, 1941, **31**, 766.

[7] Engel, R. W., and Salomon, W. D., *J. Nutrition*, 1941, **22**, 109.

‡ Generously supplied by Merck and Co., Inc., Rahway, N.J.

§ We thank S. S. Zevin for these determinations (Method of Josephs, H. W., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 112.

degrees within 2 to 10 days. The vitamin A fluorescence at first increased over the low levels in the newly weaned animals. After the third day, however, the fluorescence diminished steadily as the liver fat accumulated. Only in irregular patches, did Kupffer cells and large fat droplets of surrounding liver cells show appreciable vitamin A fluorescence. This fluorescence diminished towards the periphery of the patches. Outside of these areas, the fluorescence was completely absent. Shortly before death of the rats, such patches were very small and the Kupffer cells free from vitamin A. Thus, the total vitamin A in the liver at this time, was much less than at the start of the experiment, despite liberal carotene supplements. Livers of control animals receiving choline daily were rich in vitamin A. About the sixth day, the rats showed the characteristic hemorrhagic renal changes.[8] Marked vitamin A fluorescence appeared in the interstitium of the renal cortex in a distribution following the nephron. The adrenals were hemorrhagic in some animals, vitamin A-free in all. Ocular hemorrhage was occasionally seen in rats displaying the most severe changes. No sex difference in vitamin A distribution was apparent.

Ten rats which either spontaneously had survived the critical period of choline deficiency (10 days) or had received subnormal choline supplements were sacrificed after 20 to 90 days on the diet. Their livers were fatty with no vitamin A in Kupffer cells and only patchy traces or none at all in fat droplets. By chemical analysis, from 1.3 to 19 International Units of vitamin A per gram were found, in contrast to the normal value of over 200 I.U. Male animals showed much less vitamin A than females both chemically and histologically. The kidneys seemed normal upon routine histological examination except for scars indicating some past injury. With the fluorescence microscope, however, the cortical interstitium appeared extremely rich in vitamin A in a nephron-bound distribution.

The progeny of females on the deficient diet showed fatty livers with little vitamin A. This condition was accentuated if mother and litter were further maintained on the choline-free ration. Liver vitamin A values as low as 0.5 I.U. per gram were observed.

Liver storage of vitamin A thus depends not only upon dietary ingestion and fat deposition, but also upon choline intake. The disappearance of vitamin A from the fatty livers of choline-deficient rats is not related to the acute stage with "hemorrhagic degeneration" since it is even more marked in the chronic experiments. Nor is inability of the liver to convert carotene into vitamin A a major factor, since preliminary experiments replacing carotene by vitamin A in the diet reveal the same picture. The excessive vitamin A of the kidneys, never seen normally,[9] excludes an absorption difficulty. Furthermore, the wealth of vitamin A in histologically normal as well as hemorrhagic kidneys would seem to exclude renal pathology as a fundamental cause. Whether the vitamin A deposits in the kidney are due to compensatory storage or urinary excretion cannot yet be answered.

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[8] György, P., and Goldblatt, H., *J. Exp. Med.*, 1940, **72**, 1.

Summary. Rats on a choline-poor diet containing liberal supplements of carotene develop fatty livers poor in vitamin A. The kidneys of these animals are extremely rich in vitamin A.

13514 P

Localized Maturation of Lid-closure Reflex Mechanism by Thyroid Implants into Tadpole Hindbrain.*

JERRY J. KOLLROS. (Introduced by Paul Weiss.)

From the Department of Zoology, The University of Chicago.

The lid-closure (wink) reflex of amphibians—retraction of the eye bulb upon tactile stimulation of the cornea or surrounding skin—appears comparatively late in development. Urodeles display it first in advanced larval stages, anurans at or about the time of metamorphic climax. In the latter group its dependence upon the metamorphosing agents was demonstrated by experiments in which metamorphosis was either suppressed (hypophysectomy) or advanced (hyperthyroidism);[1] in the former case, the reflex failed to appear unless thyroid was administered, while in the latter case its onset was advanced almost, though not fully, as much as was the transformation of the body. Both the sensory (trigeminal)and motor (abducens) components of the reflex develop full functional capacity in very early larval stages, but their central linkage into a workable reflex mechanism is not effected until at the time of metamorphosis.

The close association with metamorphosis suggested that maturation of the central mechanism of the reflex might be under direct

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[9] Popper, H., and Greenberg, R., *Arch. Path.*, 1941, **32**, 11.

* This work was done under the direction of Dr. Paul Weiss. It has been supported by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago.

[1] Kollros, J. J., *J. Exp. Zool.*, 1942, **89**, 37.

control of the thyroid hormone. In order to test this possibility, an attempt was made to precipitate the development of the reflex relative to the metamorphic changes in the rest of the body by exposing the hind brain locally to supernormal concentrations of the hormone. As Hartwig[2] has shown, implantation of thyroxin-soaked agar under the fin skin of salamander larvae causes local metamorphosis of the surrounding tissues. A similar procedure was, therefore, adopted for the present experiments.

Agar pellets were soaked in thyroxin (1:1000) and then implanted into the fourth ventricle of tadpoles of *Rana pipiens*. In another series fresh rat thyroids were used. The grafts could be expected to exert both localized and diffuse effects, the latter to speed metamorphosis in general. Local effects resulting from higher concentration of the hormone at the site of the graft might register not merely in absolute precocity, but in definite advancement of the onset of the reflex relative to other accelerated body changes. Emergence of the fore limbs ("f.l.e.") was chosen as a criterion of body metamorphosis.

According to earlier observations,[1] the reflex in the normal tadpole antedates f.l.e. by about 4 days. In 29 control animals of the present series this period was found to be from 0 to 10 days (average 4.1 days). In animals precociously metamorphosed by diffuse thyroid treatment, the reflex usually lags behind f.l.e. by from 1 to 5 days. In contrast, 33 animals with thyroid implants in the fourth ventricle displayed the reflex first from 1 to 18 days (average 10.5 days) prior to f.l.e., indicating a definite local acceleration of functional brain differentiation in the vicinity of the graft. The treated animals fall into two classes according to age. The 12 youngest specimens (hind legs with beginning digitation), although initially stimulated, apparently received insufficient hormone from the implant to complete metamorphosis at an accelerated pace, and in these the reflex appeared at the usual time. If the computation is confined to the 21 cases treated at a later stage (all toes clearly outlined), the average advancement of the reflex relative to f.l.e. rises to 12.7 days (Fig. 1), with the reflex in all cases present at least 7 days prior to f.l.e. When the implants are made into animals with only an anticipated 10 or 11 days to go until f.l.e., the reflex appears over night, while the anticipated interval until f.l.e. is reduced by one-half.

Animals with thyroid grafts in the brain often exhibit striking asynchronism in the first appearance of the reflex in the 2 eyes. Whereas the 2 eyes of normal tadpoles are never more than one day

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[2] Hartwig, H., *Biol. Zent.*, 1940, **60**, 473.

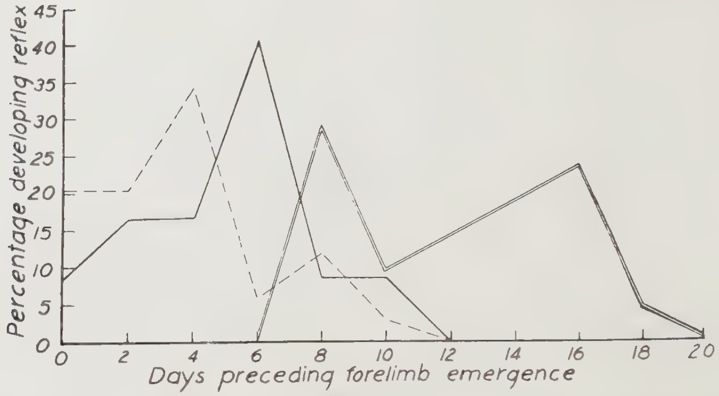

FIG. 1.

Frequency curves showing the percentage of animals developing the corneal reflex in successive 2-day intervals in the period preceding forelimb emergence; – — — normal controls, ——— animals having received thyroid implants at an earlier stage (hind limb undergoing digitation), ═══ animals having received thyroid implants at a later stage (all digits distinguishable).

apart in showing the first signs of the reflex, some of the animals with implants showed a difference between the 2 sides of as much as 7 days. This difference is interpreted as resulting from an asymmetrical position of the graft in the ventricle, subjecting the 2 lateral halves of the brain wall to different hormone concentrations. This assumption is borne out by 2 cases studied microscopically in sections, in which the grafts actually were found to be situated nearer the side which had displayed the reflex first.

In conclusion, these experiments seem to prove a direct local effect of thyroxin on the setting into operation of the brain centers mediating the lid-closure reflex. The hormone exerts this effect only in older larvae. Its ineffectiveness during mid-larval stages (save for general metamorphic acceleration) indicates that the reflex mechanism itself differentiates independently of hormone action, obviously by self-differentiation within the brain centers.[3] Only the final stages of this process and the release of the apparatus for actual operation seem to require the supplementary action of thyroid hormone.

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[3] Weiss, P., *Comp. Psychol. Monogr.*, 1941, **17**, No. 4.

13515

Streptothricin, a New Selective Bacteriostatic and Bactericidal Agent, Particularly Active Against Gram-Negative Bacteria.*

SELMAN A. WAKSMAN AND H. BOYD WOODRUFF.

From the New Jersey Agricultural Experiment Station, New Brunswick, N.J.

Most of the bacteriostatic and bactericidal substances of microbial origin that have been so far isolated act primarily against gram-positive bacteria, and only to a limited extent upon gram-negative organisms. Even those preparations, like pyocyanase and actinomycin, which act upon gram-negative bacteria are still more active against the gram-positive forms. The action of the different substances is selective both qualitatively with respect to the specific kinds of organisms affected, and quantitatively in regard to the relative concentration of the active substance required to inhibit the growth of various bacteria or to destroy them. This selective action also expresses itself in the relative toxicity of the various materials to animals and their activity *in vivo*.

A new substance can now be added to this list of antibiotic agents. This substance has been obtained from a soil *Actinomyces,* and is designated as *streptothricin,* derived from the early generic designation *Streptothrix,* given to this group of organisms. This is the fourth type of antibiotic substance produced by actinomycetes. Of the three other compounds, only one has so far been isolated in a crystalline form, namely *actinomycin.*[1] The other two are *actinomycetin,*[2, 3] a water-soluble, thermostable, protein-like preparation, which causes lysis of certain specific bacteria, the presence of the living mycelium of the antagonist being favorable to its antagonistic action, and *lysozyme,*[4, 5] a water-soluble, alcohol-insoluble, thermostable preparation, stated to be similar to the lysozyme of egg white.

The new organism was isolated† from the soil by the use of the bacteria-enriched agar-plate.[1] It is in many respects similar to a species described a quarter of a century previously as *Actinomyces lavendulæ*,[6] although it differs somewhat from it in certain respects. Streptothricin is produced by growing the organism in a tap-water medium containing 1% glucose or starch, 0.5% tryptone, 0.2% K_2HPO_4, 0.2% NaCl, 0.001% $FeSo_4$, and 0.25% agar. It is also formed when the organism is grown on a variety of simple nitrogenous compounds, such as glycocoll, alanine, aspartic acid, aspargine and glutamic acid, with small amounts of glucose, and even in the complete absence of the carbohydrate, with some of the amino acids. No growth of the organism is obtained on tryptophane, phenyl alanine and certain simple inorganic salts of nitrogen; with ammonium sulfate, growth is obtained but no active substance produced. The formation of the active substance is thus associated with the nitrogen nutrition of the organism. Iron appears to play an essential rôle in the production of the substance. An increase in growth as a result of an increase in carbohydrate concentration does not result in an increase in streptothricin content; however, an increase in growth as a result of an increase in the amino-acid concentration, with the same amount of carbohydrate, causes an increase in the concentration of the active substance. When streptothricin is produced upon an amino acid alone, in the absence of carbohydrate, there is a gradual increase in alkalinity, which results in the destruction of the substance.

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* Journal series paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Soil Microbiology.

[1] Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 609; *J. Bact.,* 1941, **42**, 231.

[2] Gratia, A., et Dath, S., *Compt. rend. Soc. Biol.,* 1924, **91**, 1442; 1925, **92**, 1125; **93**, 451; 1926, **94**, 1267.

[3] Welsch, M., *Compt. rend. Soc. Biol.,* 1937, **124**, 573.

[4] Krassilnikov, N. A., and Koreniako, A. I., *Microbiologia,* 1939, **8**, 673.

[5] Kriss, A. E., *Microbiologia,* 1940, **9**, 32.

† The isolation of this organism was carried out by Dr. W. Kocholaty of the University of Pennsylvania, while working in our laboratory.

The *A. lavendulæ* is grown upon one of the above media, for 8 to 10 days, at 28°C, and the streptothricin isolated. It is readily soluble in water and in dilute mineral acids, but is destroyed by concentrated acids. It is insoluble in ether, petrol ether, and chloroform. It is precipitated from the tryptone medium by protein-precipitants, but, upon isolation, it ceases to give proteic characteristics. This is due to the fact that it is bound in the culture to a protein, becoming inactivated upon the coagulation of the latter. In the crude culture-filtrate and in the alcohol-precipitated form, streptothricin is thermolabile, whereas, in the purified state, it is thermostable, withstanding 100°C for 15 minutes. Treatment with proteolytic enzymes does not reduce its activity. On adjusting the reaction of the medium, upon the completion of growth, to pH 3.5 with acid, a precipitate is produced, the filtrate containing virtually all the activity. Streptothricin is completely adsorbed, at neutrality, on charcoal (norit A), from which it can be removed by treatment with dilute mineral acid for 8 to 12 hours. The acid-extract is neutralized and concentrated *in vacuo*, at 50°C, just to dryness. The residue is extracted with absolute alcohol, filtered, evaporated, and taken up in water. This particular type of preparation usually contains 40 to 50% inorganic matter and 2 to 3% nitrogen, on an ash-free basis. Further concentration and reduction in ash-content were obtained by subsequent treatments. On electrodialysis, the active substance moves to the cathode at pH 7.0. In general, the properties of this substance appear to be those of an organic base.

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[6] Waksman, S. A., and Curtis, R. E., *Soil Sci.*, 1916, **1**, 99.

Streptothricin is highly active, even in crude preparations, against various gram-negative and gram-positive bacteria, in concentrations

TABLE I.
Inhibitory Effect of Streptothricin upon Growth of Various Bacteria.

Organism	Crude streptothricin added per 10 cc agar, mg					
	3	1	0.3	0.1	0.03	0.01
Bac. subtilis	0[‖]	0	0	0	0	1
Bac. mycoides	2	2	2	2	2	2
Bac. macerans	2	2	2	2	2	2
Bac. megatherium	0	0	0	0	1	2
Bac. polymyxa	0	0	2	2	2	2
Bac. cereus	2	2	2	2	2	2
Micrococcus lysodeikticus	0	0	0	1	2	2
Staphylococcus musicæ	0	0	0	1	2	2
Sarcina lutea	0	0	0	0	1	2
Aërobacter aërogenes[*]	0	0	1	2	2	2
Aërobacter aërogenes	0	0	0	Tr	2	2
Escherichia coli[†]	0	0	0	0	2	2
Escherichia coli 4348	0	0	Tr	1	2	2
Serratia marcescens	0	1	2	2	2	2
Serratia marcescens	1	1	2	2	2	2
Pseudomonas fluorescens[‡]	2	2	2	2	2	2
Shigella gallinarum	0	0	0	0	1	2
Pasteurella pseudotuberculosis	0	0	0	Tr	2	2
Salmonella cholerasuis	0	0	0	Tr	2	2
Salmonella schottmülleri	0	0	0	1	2	2
Salmonella abortivoequina	0	0	0	Tr	2	2
Salmonella typhimurium	0	0	0	2	2	2
Hemophilus suis	0	0	0	2	2	2
Hemophilus influenzæ	0	0	0	0	0	1
Brucella abortus	0	0	0	0	2	2
Azotobacter agile	0	0	0	0	0	2
Azotobacter vinelandii	0	0	0	0	0	2
Azotobacter chroococcum	0	0	0	Tr	2	2
Azotobacter indicum	0	0	0	2	2	2
Mycobacterium phlei	0	0	0	1	2	2
Clostridium butylicum[§]	2	2	2	2	2	2
Lactobacillus casei[§]	0	0	0	2	2	2
Actinomyces albus	0	0	0	1	2	2
Actinomyces violaceus-ruber	0	0	—	—	—	—
Actinomyces lavendulæ	0	1	2	2	2	2

[*] Representing 3 distinct strains.

[†] Representing 5 strains of *E. coli* obtained from different sources.

[‡] Representing 4 strains.

[§] Cultured anaërobically.

[‖] 0 = no growth, 1 = limited growth, 2 = good growth, Tr = trace of growth.

of 0.1 mg crude material per 10 cc agar, as shown in Table I. There is very little difference in the degree of sensitivity between *E. coli* and *S. lutea; B. subtilis* is more sensitive than either of those, whereas *Bac. mycoides* is not sensitive at all. Various spore-forming bacteria are thus found to show marked differences in the degree of sensitivity to this substance.

Streptothricin also possesses strong bactericidal properties, especially for various gram-negative bacteria (Table II). However, higher concentrations are required than for its bacteriostatic action.

Summary. A new type of antagonistic substance, designated as streptothricin, has been obtained from a soil *Actinomyces.* The substance appears to be formed from certain amino acids in the medium. Streptothricin is insoluble in ether, petrol ether or chloroform, and is carried down by precipitating the medium with ethyl alcohol. It is best separated from the medium by adsorption on norit-A, followed by elution with dilute mineral acid. Further concentration is accomplished by neutralization of the acid and concentration *in vacuo.* The active substance is low in nitrogen, is not affected by proteolytic enzymes and can withstand 100°C, for 15 minutes. Its properties are those of an organic base.

The bacteriostatic action of streptothricin is unique. The addition of 0.1 mg of crude material to 10 cc of nutrient agar inhibits the growth of *E. coli* and various other gram-negative bacteria; among the gram-positive bacteria, some, like *Bac. subtilis* and micrococci, are more sensitive, whereas others, like *Bac. mycoides,* are far more resistant. Streptothricin possesses also marked bactericidal properties, especially upon certain gram-negative bacteria.

TABLE II.
Bactericidal Action of Streptothricin upon *E. coli* and *B. subtilis.*
Washed suspension of bacteria in 10 ml sterile water.

	E. coli		*M. lysodeikticus*	
Bacteria at start, per 1 cc	500,000,000		17,600,000	
	Number of viable cells per 1 cc left after			
Streptothricin, mg	17 hr × 1000	65 hr × 1000	17 hr × 1000	65 hr × 1000
0	450,000	413,000	1,900	393
0.01	430,000	362,000	800	82
0.1	290,000	213,000	500	10
1.0	2,400	18	100	5
5.0	1,000	7	—	—

13516

Folic Acid in the Growth of *Cl. tetani.*

J. HOWARD MUELLER AND PAULINE A. MILLER.
*From the Department of Bacteriology and Immunology, Harvard Medical School, and School of Public Health, Boston, Mass.**

Recent work in this laboratory[1] has demonstrated the requirement by a strain of *Cl. tetani* for a component of liver extract originally described in another connection by Snell and Peterson[2] and subsequently studied by Stokstad.[3] This material, together with thiamine and riboflavine, fully accounted for the activity of a fraction of liver extract which had been found essential to the growth of our tetanus organism. The active component of Snell and Peterson's fraction has been further investigated by Mitchell, Snell and Williams,[4] who have carried the purification considerably further, and have named the agent "folic acid" since they find green leaves to be a convenient source. Its chemical constitution has not yet been determined.

Through the courtesy of Professor Williams it has been possible to test his purified material as to its growth-promoting effect on the tetanus bacillus. Three specimens, representing varying degrees of concentration have been made available. The amounts required to produce half maximum growth of *L. helveticus* were given as follows:

Specimen	1	6.0 gamma
,,	2	0.005 ,,
,,	3	0.0016 ,,

These preparations were used in varying quantities to replace the Snell and Peterson or Stokstad factor in an otherwise complete medium.[1] After seeding with our strain of *Cl. tetani* and anaerobic incubation for 24 hours, relative growths were determined in a photoelectric nephelometer. The results, expressing degree of growth approximately as percentage of maximum are shown in Table I.

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* Aided by a grant from the Commonwealth Fund.

[1] Mueller, J. H., and Miller, P. A., *J. Bact.*, in press.

[2] Snell, E. E., and Peterson, W. H., *J. Bact.*, 1940, **39**, 273.

[3] Stokstad, E. L. R., *J. Biol. Chem.*, 1941, **139**, 475.

[4] Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1941, **63**, 2284.

TABLE I.

Tube No.	Folic acid concentrate, specimen	Amt per cc, γ	% of max. growth
1	I	50.0	100
2	”	25.0	100
3	”	10.0	100
4	”	5.0	70
5	”	2.5	40
6	”	1.0	20
7	II	.025	100
8	”	.01	100
9	”	.005	100
10	”	.0025	100
11	”	.001	60
12	”	.0005	10
13	”	.00025	trace
14	III	.01	100
15	”	.005	100
16	”	.0025	100
17	”	.001	70
18	”	.0005	40
19	”	.00025	30
20	”	.0001	25
21	none		trace

It is evident that "folic acid" is essential to the growth of this strain of tetanus, and that its effect is exerted by extremely minute quantities.

13517

Nature of Hydrosulphosol and Its Toxicity to Living Embryonic Tissue.

A. J. SALLE AND M. KORZENOVSKY.

From the Department of Bacteriology, University of California, Los Angeles.

Compounds containing the sulfhydryl (–SH) radical have been shown to be of great practical value in promoting the healing of wounds. Glutathione is probably the best known compound of this type which is present in living tissues. It is of almost universal occurrence in animal tissues where the concentration has been found to roughly parallel the intensity of metabolic activity. The concentration is unusually high in regenerating or embryonic tissues. Voegtlin, Johnson and Thompson[1] reported that malignant tumor cells showed higher concentrations of glutathione than normal tissue cells. Therefore, it appears that one of the important biological functions of glutathione, as well as other compounds of a similar nature, is related to growth.

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[1] Voegtlin, C., Johnson, J. M., and Thompson, J. W., *Public Health Rep.*, U. S. Public Health Service, 1936, **51**, 1689.

Reimann[2] employed thiocresol in a concentration of 1:10,000 as a wet dressing on a number of stubborn wounds and reported a stimulation of cell division. On the basis of the results obtained from the use of thiocresol, and a number of other compounds containing the –SH group and their homologues containing other groups, he concluded that the sulfhydryl radical was the effective agent. Riley[3] used a lanolin ointment containing cysteine hydrochloride on skin reactions following deep X-ray or radium therapy and believed that the healing was hastened by the presence of the –SH radical.

Hydrosulphosol* is an available and convenient source of –SH radicals. This fact, together with its value in ultraviolet burns, was discovered by Dr. Ralph R. Mellon (McBroom[4]) and his associates at the Western Pennsylvania Hospital's Institute of Pathology in Pittsburgh. As a result they suggested that it receive a thorough clinical trial in thermal burns. During the past year about 150 chemical and thermal burns have been treated by Dr. W. F. Pierce[5] and by Dr. A. E. Cruthirds.[6] About two-thirds of these have been serious, either in degree (second or third), or in extent, or both. The results have been so highly satisfactory as to justify the increasing clinical use that the solution is now receiving.

In addition, Hydrosulphosol contains the pentathionate ion in

TABLE I.
Chemical Analysis of Hydrosulphosol

Specific gravity	1.032
pH of solution (glass electrode)	10.2
Solids remaining after evaporation of 100 g solution to constant weight	10.56

Constituent	%
Calcium from calcium oxide	0.549
Total sulfur (iodine titration)	1.46
Sulfur as sulfide ion	0.30
'' '' thiosulfate ion	0.22
'' from polysulfide	1.256
Sulfhydryl ion (methylene blue titration)	0.237

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[2] Reimann, S. P., *J. A. M. A.*, 1930, **94**, 1369.

[3] Riley, J. F., *Brit. Med. J.*, 1940, **2**, 516.

* Hydrosulphol is a liquid preparation supplied by the Earl Hill Wilson Foundation for Medical Research, Los Angeles, California.

[4] McBroom, J., *Med. Rec.*, 1940, **152**, 299.

[5] Pierce, W. F., *Am. J. Surg.*, 1941, **53**, 434.

[6] Cruthirds, A. E., personal communication.

compounds of polysulfides and thiosulfates in aqueous solution. The composition of the solution is given in Table I. Because of the nature of the solution it was not possible to obtain a more accurate analysis. On the addition of distilled water colloidal sulfur is rapidly precipitated indicating that the solution is unstable chemically. Available evidence points to the fact that the pH drops when the solution comes in contact with organic matter, as occurs in burns.

Methylene blue may be used as a test for compounds containing the –SH radical. The dye is rapidly decolorized in the presence of the sulfhydryl group and other reducing substances. The H of the sulfhydryl group readily combines with methylene blue, reducing it to the colorless compound. This is the only group present in Hydrosulphosol which was found to be capable of reducing methylene blue. The amount of methylene blue decolorized indicates that Hydrosulphosol contains about 0.237 g of the –SH ion per 100 cc. This is approximately 50 times the concentration of the ion present in human blood.

Another test for the presence of sulfhydryl-containing compounds is their ability to support growth of anaerobic organisms under aerobic conditions. Ten tubes of nutrient broth, each containing 10 cc, were heated in an Arnold sterilizer for 10 minutes to remove dissolved oxygen. To one series of 5 tubes were added 0.05 cc, 0.1 cc, 0.2 cc, 0.3 cc, and 0.4 cc Hydrosulphosol respectively. The other series served as a control. The tubes were each inoculated with one drop of a culture of *Clostridium sporogenes* and incubated at 37°C for 48 hours. A similar series was set up employing *Clostridium perfringens*. At the end of 48 hours the control tubes in both series showed no growth, whereas the tubes containing Hydrosulphosol showed luxuriant growth. An amount of Hydrosulphosol as small as 0.05 cc was sufficient to yield a heavy growth of the organisms in the presence of air. Smaller amounts were not tried.

These facts have stimulated investigations of Hydrosulphosol in the treatment of burns. Since it is being employed to an ever increasing extent it was considered of great interest and importance to see what effect the solution would have on living embryonic tissue fragments cultivated *in vitro*.

Accordingly, a series of dilutions of Hydrosulphosol were prepared in physiological salt solution. To 4 cc of each dilution was added 1 cc of a mixture of 3 parts of embryonic extract and 1 part of horse serum as a source of organic matter, in an endeavor to duplicate actual clinical conditions. The final dilutions of Hydrosulphosol were 1:20, 1:40, 1:60, 1:80, and 1:100. Five cc of each dilution were pipetted into sterile test tubes and placed in a 37°C water bath. Embryonic chick hearts were cut into fragments about 0.5 mm in diameter. About 100 fragments were pipetted into 5 cc of each dilution of Hydrosulphosol. At the end of 10 minutes the fragments were thoroughly washed and embedded in plasma contained in Carrel flasks. Slight growth occurred in the 1:20 dilution after an incubation period of 48 hours and normal growth occurred in a dilution of 1:80. It is believed that the toxicity of the compound to tissue was due to its high alkalinity. In the higher dilutions the alkalinity was sufficiently reduced to permit the growth of tissue. The original solution could not be adjusted to a lower pH owing to the fact that a rapid and heavy precipitate appeared on the addition of acid.

In another test a series of higher dilutions of Hydrosulphosol were prepared and allowed to remain in contact with the embryonic chick heart tissue fragments for 72 hours instead of only 10 minutes. The test was performed as follows: Tissue fragments were embedded in plasma contained in Carrel flasks. After the plasma had coagulated the surface was flooded with Hydrosulphosol dilution. One cc of Hydrosulphosol dilution was added to 2 cc of the coagulated plasma mixture (plasma-Tyrode solution-embryonic extract-tissue fragments). The dilutions ranged from 1:160 to 1:800. Although growth occurred in all dilutions only those from 1:400 and above showed proliferation as luxuriant as the controls. In the higher dilutions there was no evidence that stimulation beyond normal growth occurred. It must be remembered that chick heart tissue was used instead of skin. Tests are now under way using only embryonic skin for the tissue fragments.

Conclusions. Evidence has been presented to show that Hydrosulphosol is an available and convenient source of –SH radicals. The compound rapidly decolorizes methylene blue and supports the growth of the anaerobic organisms *Clostridium sporogenes* and *Clostridium perfringens* under aerobic conditions. The compound is relatively nontoxic to embryonic chick heart tissue fragments cultivated *in vitro*.

13518

Reduction of Toxicity of 2-Sulfanilamidopyrimidine (Sulfadiazine) by Phenylazo-alpha-alpha-diaminopyridine Hydrochloride (Pyridium) in the Mouse.

OSCAR BODANSKY.

From the Department of Pharmacology, Cornell University Medical College, New York.

The possibility of antagonism in systemic toxicity between the sulfonamides and other compounds has begun to receive attention during the past year. McCarty[1] found that para-aminobenzoic acid has no effect on the acute fatal toxicity of sulfapyridine in mice. David, *et al.*,[2] reported that pentobarbital exerted a protective action against the toxicity of intravenously administered heparin-sodium sulfapyridine mixtures in dogs. Richards[3] found that small doses of urethane or pentobarbital decrease the fatal toxicity of intravenously administered sodium sulfapyridine in rabbits and dogs. In the present paper it is shown that the acute toxicity of 2-sulfanilamidopyrimidine (sulfadiazine) in the mouse is reduced by phenylazo-alpha-alpha-diaminopyridine hydrochloride (pyridium). The toxicity of sulfanilamide is not affected by this compound.

The percent mortality in a group of mice injected subcutaneously with a given dose of sulfonamide was compared, under the same experimental conditions, with the mortality in groups receiving this dose and, in addition, doses of 10, 30 or 100 mg of pyridium per kg. For each experiment, 3 groups consisting of 8 to 15 mice each were employed. A total of 269 mice was used in the experiments here reported. The sodium salts of sulfadiazine and sulfanilamide* were injected subcutaneously in concentrations of 5 to 10%. Pyridium was injected in a 0.2% solution or a 0.5% suspension. When both sulfonamide and pyridium were employed, they were injected within a few seconds of each other into widely separated sites. The mice were given water and food *ad lib.* and the number of deaths occurring in 4 days noted.

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[1] McCarty, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 133.

[2] David, N. A., Phatak, N. M., Donnell, H., and Vers, H., *J. Am. Pharm. Assn.*, 1941, **30**, 38.

[3] Richards, R. K., *J. Lab. and Clin. Med.*, 1941, **26**, 1256.

* The sodium salts were prepared according to the directions of Marshall, Bratton and Litchfield.[4] Sodium sulfadiazine, furnished through the courtesy of the Nepera Chemical Co., was also employed.

[4] Marshall, E. K., Jr., Bratton, H. C., and Litchfield, J. T., Jr., *Science*, 1938, **88**, 597.

The results are shown in Table I. Experiment 1 is a control, showing that pyridium in doses of 30 and 100 mg per kg did not cause any deaths ascribable to the drug. Walton and Lawson[5] found a 20% mortality in rats receiving 200 mg of pyridium per kg intravenously, and a 100% mortality at a dose of 450 mg per kg.

Experiments 2, 3, and 4 show that 1.5 g of sulfadiazine led to a mortality of from 80 to 100%. This dose of sulfadiazine together with doses of 30 or 100 mg of pyridium per kg resulted in a much lower percent mortality, the higher dose appearing somewhat more effective in this respect. Thus, in experiment 4, a dose of 100 mg of pyridium per kg decreased the mortality from 93 to 25%. All 3 experiments showed this reduction in mortality.

Since the percent mortality in the preceding experiments was

TABLE I.
Effect of Pyridium on Toxicity of Sulfonamides.

Pyridium, mg/kg	No. of mice	No. dead in 4 days	Mortality, %
Exp. 1. Control—No Sulfonamide.			
0	10	1	10
30	10	1†	10
100	15	1†	7
Exp. 2. 1.5 g Sodium Sulfadiazine per kg.			
0	10	8	80
30	10	3	30
100	10	3	30
Exp. 3. 1.5 g Sodium Sulfadiazine per kg.			
0	10	10	100
30	10	9	90
100	14	9	65
Exp. 4. 1.5 g Sodium Sulfadiazine per kg.			
0	15	14	93
30	15	12	80
100	12	3	25
Exp. 5. 1.2 g Sodium Sulfadiazine per kg.			
0	10	5	50
30	10	1	10
100	10	0	0
Exp. 2a. 2.0 g Sodium Sulfanilamide per kg.			
0	10	7	70
10	9	4	44
30	8	3	38
100	11	8	73
Exp. 3a. 2.0 g Sodium Sulfanilamide per kg.			
0	10	8	80
30	10	6	60
100	10	9	90
Exp. 4a. 2.0 g Sodium Sulfanilamide per kg.			
0	10	5	50
10	10	5	50
30	10	6	60

†These mice were found to have been eaten by the others in the cage, and probably do not represent pyridium toxicity.

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[5] Walton, R. P., and Lawson, E. H., *J. Pharm. and Exp. Therap.*, 1934, **51**, 200.

higher than that which Feinstone, *et al.*,[6] have reported for a dose of 1.5 g of sulfadiazine per kg (43.5%), it was decided to determine the effect of pyridium on the toxicity of a smaller dose of sulfadiazine. Experiment 5 shows that the mortality at a dose of 1.2 g of sulfadiazine per kg was 50%. The simultaneous administration of this dose and 30 mg of pyridium per kg reduced the mortality to 10%, and when the sulfadiazine and 100 mg of pyridium per kg were given, no deaths resulted.

In contrast to the effect of pyridium on the toxicity of sulfadiazine, this substance had no effect on the toxicity of sulfanilamide, as may be seen from examination of experiments 2a, 3a, and 4a.[‡]

13519 P

Increase in Weight of Gonads Following Injection of Single Male Rat Pituitary.[*][†]

R. P. REECE AND E. J. WEATHERBY.

From the Department of Dairy Husbandry, New Jersey Agricultural Experiment Station, New Brunswick, N.J.

When increases in ovarian weight of the sexually immature rat have been employed in determining the gonadotropic content of the rat pituitary it has been necessary to implant[1] or inject[2] more than

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[6] Feinstone, W. H., Williams, R. D., Wolff, R. T., Huntington, E., and Crossley, M. L., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 427.

[‡] The writer is indebted to Dr. W. J. Youden of the Boyce Thompson Institute for Plant Research for a statistical analysis of these results. The alive : dead ratio of all the mice in experiments 2 to 5 should be 59:77 if the pyridium had no effect on the toxicity of the sulfadiazine. Calculation of chi square from the differences between the calculated expectations and the mortalities at the concentrations of 0, 30 and 100 mg of pyridium per kg yields a value of 22.82. "a value highly improbable if the ratio were really the same for all concentrations." An analysis of the variance showed that, if the pyridium were really without effect, the decrease in the toxicity of the sulfadiazine obtained would occur *by chance* only once in something like one hundred instances, and certainly not more often than once in fifty.

[*] Journal Series Paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Husbandry.

[†] This study has been aided in part by a grant from the National Dairy Products Corporation.

[1] Evans, H. M., and Simpson, M. E., *Am. J. Physiol.*, 1929, **89**, 371.

[2] Hellbaum, A. A., and Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 902.

one pituitary gland. This technic requires a large number of donor rats and therefore does not readily lend itself to the extensive study of factors influencing the gonadotropic content of the rat pituitary gland. Our observations indicate that increases in ovarian weight of sexually immature rats can be obtained by injecting a single male rat pituitary gland.

Pituitary glands were collected from 80 sexually mature male rats, and each pituitary was macerated separately by means of an agate mortar and pestle. One drop of .1 N NaOH was added and the macerated tissue taken up in 3.2 cc of distilled water. Four-tenths of one cc of this suspension was injected subcutaneously twice daily for 4 days into a 24- to 26-day-old female rat. The recipient was killed 100 hours after the first injection, and the ovaries were weighed. For controls the ovaries of fifty-seven 28- to 30-day-old non-injected rats were weighed. Body weighings were made on the first day of injection and at the time of autopsy.

Body weights of the control and experimental rats were similar on the first injection day, 47 and 46 g respectively, and at the time of autopsy, 53 and 51 g respectively. Average ovarian weight of the control animals was 13.4 mg, whereas that of the injected animals was 54.8 mg. Ninety percent of the injected animals showed at least a 50% increase in ovarian weight over that of the controls. The percentage of animals showing greater increases in ovarian weight decreased at a constant rate until only 50% showed as much as a 250% increase. Above the 250% increase in ovarian weight the percentage of rats responding decreased at an irregular rate.

Preliminary observations with the day-old White Leghorn chick indicate that its testes will respond to a single male rat pituitary gland. Sixteen pituitary glands from sexually mature rats were injected subcutaneously twice daily for 4 days into 16 one-day-old chicks. The chicks were killed on the afternoon of the day following the last injection and their testes weighed. Thirty chicks receiving no treatment served as controls. Average testicular weights of the control and of the injected chicks were 9.5 mg and 15.4 mg respectively. Fifty-six percent of the injected chicks showed at least a 50% increase in testicular weight.

Summary. The ovaries of 24- to 26-day-old rats and the testes of day-old White Leghorn chicks showed an increase in weight following the injection of a suspension of a single male rat pituitary gland in 8 divided doses over a 4-day period. The average increase in ovarian weight was 309% and that of the testes was 62%.

13520

Strength-Duration Curve of Nerve Excitation by Electrical Fields Associated with Discharge of Condensers.

J. A. Gengerelli and N. J. Holter.

From the University of California at Los Angeles.

In a previous report[1] it was shown that when the sciatic of the frog is placed between the plates of a condenser separated by a distance of 7 inches, it is stimulable by the electrical field associated with a 60 ~ potential difference of 10,000 volts between them.

In the present experiments the plates were 7″ x 5″ and were separated by a distance of 2 5/16 inches. These plates were connected in parallel with condensers of various values and the system charged by a DC power supply capable of yielding a maximum potential difference of 2,000 volts.

The preparation, consisting of the sciatic and the attached gastrocnemius, was laid upon a carefully cleaned glass slide between the large plates of the condenser system in such wise that the nerve was parallel to the electrical lines of force of the field, and the long axis of the muscle at right angles to them. The edges of the glass slide were each 3 mm from the plates of the condenser. To keep the preparation from becoming desiccated a strip of cloth 3 mm wide was laid across the nerve some 2 mm from the cut end and moistened with Ringers.

It was found possible to stimulate the nerve both during the charging of the condenser as well as during the discharge. The thresholds were not equal, however, and their value was determined by altering a non-inductive resistance which was connected in series with the condenser until the gastrocnemius ceased to contract. It was found by this means that stimulation was more readily obtained on the discharge of the condenser than on the charge.

The determination of the strength-duration curve was made with the discharge of the condenser, and we utilized both varying capacities and varying resistances. The preparation was arranged as shown in the legend of Table I. The table gives the results from 5 series of experiments. In the first 4 series the resistance is fixed and the capacity is varied; in the fifth series the capacity is fixed and the resistance is varied. The values of E represent averages of from

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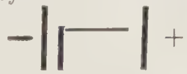
[1] Gengerelli, J. A., and Holter, N. J., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 532.

TABLE I.

C	R	CR	E	No. of determinations
.00025	20	.0050	1675	6
.00033	”	.0066	1487	
.00050	”	.010	1312	
.0009	”	.018	1237	
.0066	”	.132	1162	
.0196	”	.392	1312	
.0909	”	1.818	1650	
.10	”	2.00	1675	
.00025	”	.0050	1093	8
.00033	”	.0066	888	
.00050	”	.010	791	
.0009	”	.018	769	
.0066	”	.132	719	
.0196	”	.392	894	
.0909	”	1.818	1625	
.10	”	2.00	1831	
.00025	”	.005	1894	10
.00050	”	.010	1350	
.0050	”	.10	1156	
.01	”	.20	1144	
.05	”	1.00	1792	
.10	”	2.00	1925	
.00025	”	.0050	1063	8
.00050	”	.01	869	
.0050	”	.10	791	
.01	”	.20	831	
.0196	”	.392	1013	
.05	”	1.00	1656	
.10	”	2.00	1900	
.0099	20	.198	1266	8
”	40	.396	1325	
”	60	.594	1300	
”	80	.792	1345	
”	100	.990	1326	
”	500	4.950	1377	
”	1000	9.90	1456	
”	1950	19.30	1550	
”	5000	49.50	1725	

C is expressed in microfarads; R in ohms. To obtain CR in mscs, it is necessary to multiply the values in the table by 10^{-3}. The orientation of the preparation,



6 to 10 readings with not less than 2 preparations for each experiment; these were taken by the technic of reversed series, in order to eliminate the influence of any systematic time factor.

It will be noted from the first 4 segments of the table that the strength-duration curve presents an interesting inversion. The results of these experiments are shown graphically in Fig. 1. The locus of the inversion region is uniformly indicated by x's. It will be observed, furthermore, that the fifth experiment did not yield sufficiently small values of CR to include the inversion, hence the obtained strength-duration curve shows E as an increasing negatively

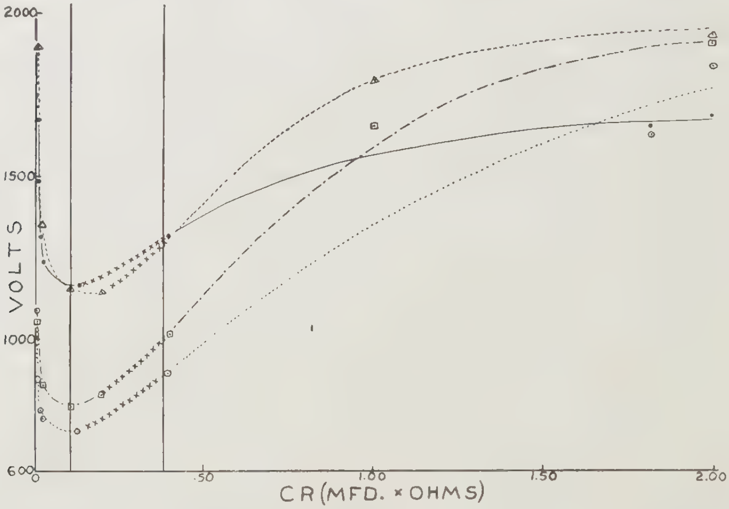


FIG. 1.

Graphs giving the results of 4 series of experiments (the first four segments of Table I). The inversion point may be considered as occurring between .0001 and .00038 mscs. These limits are indicated by the two vertical lines.

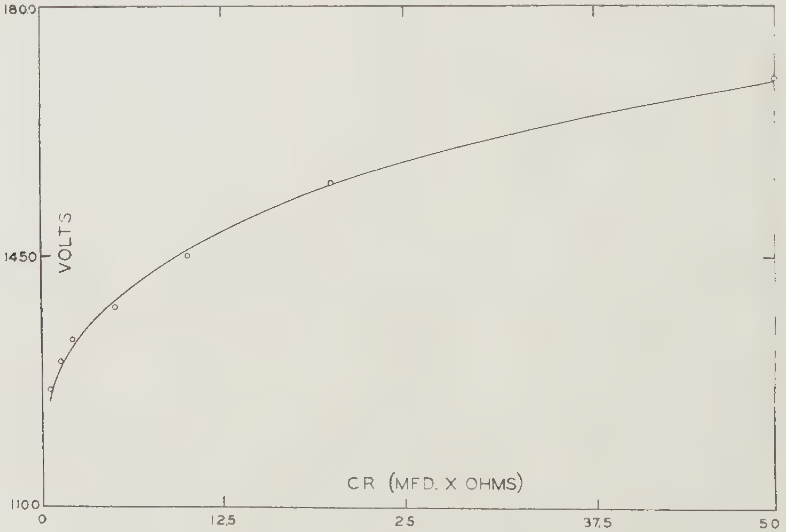


FIG. 2.

The graph illustrates the fifth segment of Table I. The experimental values for R=40 and R=80 are not shown in the graph.

accelerated function of the time. This is shown graphically in Fig. 2. The two irregularities occurring at 40 and 80 ohms, respectively, are not indicated in the curve.

Summary. 1. Stimulation of nerve by electrical fields associated with the charging and discharging of condensers has been obtained, although the efficacy of increasing and decreasing fields is not identical; 2. a strength-duration curve of excitation with diminishing electrical fields associated with the discharge of condensers has been determined wherein E is at first a decreasing and then an increasing negatively accelerated function of the time. The region of inversion is between .0001 and .00038 mscs; 3. the curve is in marked contrast with those obtained by discharging condensers through nerve tissue, in that these (a) are not characterized by an inversion, (b) show E as a *diminishing* negatively accelerated function of the time;[2] 4. there are great differences in the stimulability of preparations.

13521

An Automatic Electron Tube Stimulator and Recorder.*

H. V. CONNERTY AND W. H. JOHNSON. (Introduced by T. Koppanyi.)

From Georgetown University, School of Medicine, Washington, D.C.

As a result of the development of radio apparatus and of new types of vacuum tubes, it became possible to devise stimulators having desirable features impossible to inductorium or neon tube stimulators, *i. e.,* greater range of frequency of stimulation, greater range of intensity of each shock, and methods for recording the frequency of the stimulation. Many instruments, incorporating some or all of these features, have been devised, outstanding examples being those of Scheminzky,[1] Schmitt,[2] Nicolai,[3] and Bouman.[4] Although these instruments can be considered as great improvements over earlier types, they all have one or more of the following undesirable features: (1) large, complicated and expensive, (2) operate on batteries, (3) unwanted and irregular leakage currents, and (4) the wave form of the stimulating current is not independent of frequency.

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[2] Katz, B., *Electrical Excitation of Nerve,* Oxford Press, 1939, p. 5.

* The authors are indebted to Dr. Reginald A. Cutting, director of the Department of Physiology, for the use of his personal electrical apparatus and for stimulating the investigation in many ways.

[1] Scheminzky, F., *Arch. f. d. ges. Physiol.,* 1930, **225**, 303.

[2] Schmitt, H. A., and F. O., *Science,* 1932, **76**, 328.

[3] Nicolai, L., *Pflügers Archiv.,* 1930, **225**, 131.

[4] Bouman, H. D., *Arch. Neérl. Physiol.,* 1935, **20**, 155.

A recent instrument which is especially noteworthy for its portability is that devised by Cullen.[5] The attached signal magnet, however, always vibrates at a frequency of 60 times per second and so provides no means of indicating the frequency of stimulation. Furthermore, after having used this particular instrument the authors have found that the high amperage delivered to the signal magnet considerably shortens its life when the ordinary classroom type is used. Another undesirable feature is the presence of "stray charges in the instrument" which renders it unfit for precision work.

The authors have designed a portable (operating on 110 volts, A.C.) and inexpensive stimulator which does not have the disadvantages discussed and enumerated above. This is mainly accomplished by (1) the introduction into the oscillator circuit of a current limiting tube (type 6SK7) which takes the place of the variable resistor in the relaxation oscillator circuit of other stimulators; the resistance of this tube is a function of the control grid and screen grid voltages—the less the screen voltage and the more negative the control grid, the higher the resistance; it gives a much better control of frequency both as to range of coverage and at very low frequencies, and (2) isolating the oscillator from the output circuits by interposing a beam power tube amplifier which prevents interaction of the output circuits on the oscillator, resulting in more stable output—a result which is further assured by using inverse feedback in the amplifier circuit. Another new feature is the use of a "tuning indicator tube" (type 6E5) which blinks synchronously with each stimulus and so provides a means of visually indicating the frequency of stimulation which can be set to the chosen rate before applying the stimulating current to the tissue and signal magnet. Other features of the instrument are: (1) it is automatic; (2) it generates an ideal type of stimulating current; (3) the frequency of stimulation can be varied at will from a single shock every six seconds to a maximum rate of 2500 per second; slower or faster rates can be effected by changing the capacity of condenser C_1 or C_2; (4) the intensity can be varied without affecting the frequency, each being adjusted independently by their respective control knobs; (5) provision is made for the operation of a signal magnet which records each shock synchronously; (6) the intensity of the stimulating shocks may be adjusted to any value from zero to a maximum of ca 20 volts.

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[5] Cullen, A. B., *Science,* 1941, **94**, 219.

Details of the circuit are shown in Fig. 1. The wave form of the stimulating current is shown in Fig. 2; each stimulating shock is seen to consist of a single momentary unidirectional current.

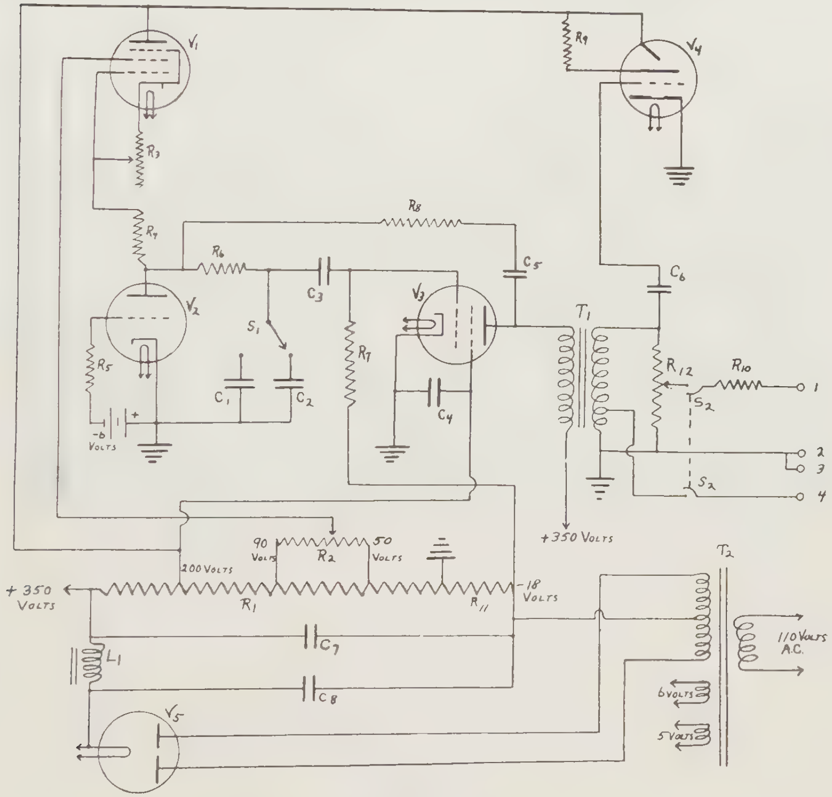

FIG. 1.

Circuit of Automatic Electron Tube Stimulator and Recorder.

V_1	Type 6SK7 tube.	R_{11}	200 ohms, 25 watt, variable.
V_2	” 884 ”	R_{12}	5,000 ” potentiometer.
V_3	” 6L6 ”	C_1	0.5 Microfarad
V_4	” 6E5 ”	C_2	0.01 ”
V_5	” 83 ”	C_3	0.5 ”
R_1	20,000 ohms, 75 watts.	C_4	1.0 ”
R_2	10,000 ohms potentiometer.	C_5	0.015 ”
R_3	3 megohms ”	C_6	0.00001 ”
R_4	50,000 ohms, 1 watt.	C_7	10.0 ”
R_5	75,000 ” 1 ”	C_8	10.0 ”
R_6	500 ” 1 ”	T_1	Output transformer.
R_7	1 megohm 1 ”	T_2	Power ”
R_8	1 ” 1 ”	L_1	10 to 20 Henry choke.
R_9	1.5 ” 1 ”	S_1	S.P.D.T. switch.
R_{10}	1000 ohms, 1 ”	S_2	D.P.S.T. ”

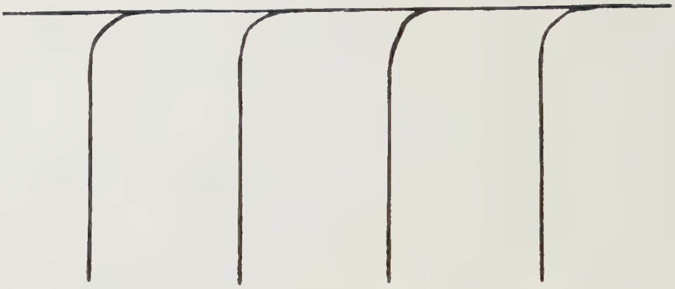

FIG. 2.

Tracing of the form of the stimulating current as visualized on the screen of an oscilloscope.

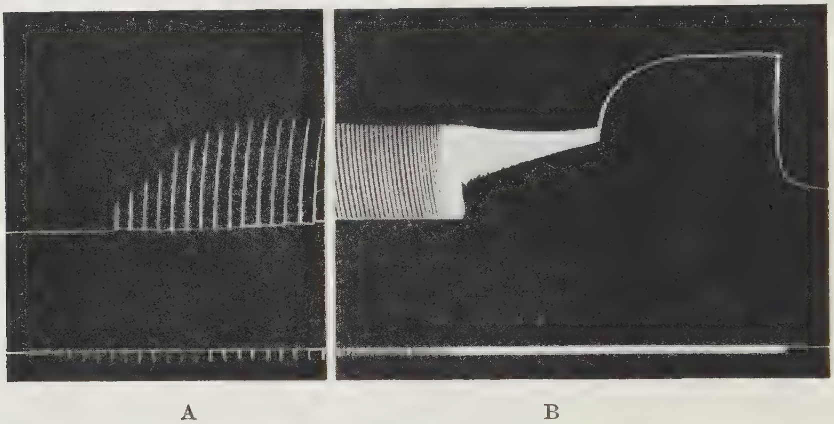

FIG. 3.

Responses of a frog's gastrocnemius muscle to different (A) intensities and (B) frequencies of stimulation.

Actual responses of a frog's gastrocnemius muscle to different intensities and frequencies are shown in Fig. 3. Note that the signal magnet works in complete unison with the stimulating current.

13522

Prevention by Certain Steroids of Testicular Atrophy Usually Elicited by Small Doses of Testosterone.*

HANS SELYE AND S. ALBERT.

From the Department of Anatomy, McGill University, Montreal, Canada.

An important argument against the clinical use of testoids[†] in patients suffering from eunuchoidism is that such compounds may aggravate the testicular atrophy when they are given in doses just sufficient to restore the accessory sex organs to the normal size. It is known that gonad atrophy is produced only with moderate doses which suffice to inhibit the hypophyseal gonadotropic hormone production, but not to exert a direct gonadotropic effect. Very high doses—which maintain the spermatogenic epithelium even in the absence of the pituitary—cause no testis atrophy in the intact organism since their direct gonadotropic effect fully compensates for the inhibition of hypophyseal function.[1] However, such high doses could hardly be given in clinical cases of eunuchoidism since they would lead to an excessive development of the secondary sex characteristics. As it has recently been shown that the testis atrophy caused by estradiol can be inhibited by testosterone and progesterone[2, 3] the question arose whether the testis atrophy normally elicited by "therapeutic doses" of testosterone might not in its turn be inhibited by steroids devoid of testoid activity. It was felt that if this could be accomplished testosterone might be given in combination with such gonadotropic steroids without the fear of aggravating testis atrophy or having to give enormous doses which would lead to excessive masculinization.

In order to study this problem 4 groups each consisting of 6 male albino rats weighing between 105 and 145 g (average 121 g) were used. Group I acted as untreated controls; Group II received 1.0 mg

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* The expenses of this investigation were defrayed by the Blanche E. Hutchinson Fund of McGill University. The authors are greatly indebted to Drs. G. Stragnell and E. Schwenk of the Schering Corporation of Bloomfield, New Jersey, for supplying all the steroids used for this work.

† This term is used as synonymous with "male hormone-like" or "androgenic" in accordance with the recently proposed pharmacological classification of the steroid hormones.[4]

[1] Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 142.

[2] Selye, H., *Canad. Med. Assn. J.*, 1940, **42**, 113.

[3] Albert, S., in press.

[4] Selye, H., *Nature*, 1941, **148**, 84.

of testosterone daily; Group III received daily 1.0 mg of testosterone and 10.0 mg of Δ^5-androstene-3(β),17(α)-diol (m.p. 182-183°C), which will henceforth be referred to as androstenediol; Group IV received 1.0 mg of testosterone and 10.0 mg of 17-ethyl-Δ^5-androstene-(β)-ol-20-one (m.p. 186°C) which will henceforth be referred to as pregnenolone. The daily dose was administered in 2 subcutaneous injections of 0.1 ml each in all cases. Testosterone was dissolved in peanut oil (5 mg/ml) while pregnenolone and androstenediol were given as finely ground crystals suspended in the same material (50 mg/ml). All animals were killed and their gonads weighed on the day following the tenth day of treatment. The average testis weight in the controls was 2.138 g and under the influence of testosterone alone declined to 1.418 g. In the group receiving androstenediol the testicular atrophy otherwise produced by testosterone was apparently inhibited since the average testis weight was 1.925 g. In the group receiving pregnenolone the atrophy was completely prevented, the average testis weight being 2.174 g.

The significance of the apparent differences between the testis weights of the testosterone-treated rats and the controls was evaluated by "Student's" method for small samples and was expressed in terms of probability estimated by graphic interpolation in Fisher's[5] table of t. It is generally agreed that the differences may be regarded as significant if P is smaller than 0.05. In this case P was smaller than 0.01. Similarly, the testes of the groups receiving androstenediol and pregnenolone respectively were significantly larger than those receiving testosterone alone, since in both cases the increase in testis weight in comparison with the last mentioned group gave a P of less than 0.01.

Since androstenediol possesses a weak testoid activity its gonad-protecting effect might be regarded as, at least partly due to this property. Pregnenolone, however—as our earlier experiments on castrate rats have shown—is devoid of any demonstrable testoid action.

Summary and Conclusions. Experiments on post-pubertal albino rats indicate that androstenediol and pregnenolone prevent the testis atrophy otherwise produced by small doses of testosterone. Since androstenediol possesses only a weak "male hormone" or testoid action and pregnenolone is completely devoid of it, it is evident that the gonad-protecting power of a steroid compound is independent of its testoid action. The experiments suggest, furthermore, that by combining moderate doses of testosterone with a non-testoid compound such as pregnenolone, therapeutic doses of testosterone may be given in cases of eunuchoidism without the danger of producing testis atrophy.

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[5] Fisher, R. A., *Statistical Methods for Research Workers*, 6th Edition, Edinburgh, 1936, p. 128.

13523 P

Comparative Distribution and Retention of Crystalloid and Colloid Fraction of Arsphenamine and Neoarsphenamine.

HAROLD N. WRIGHT AND F. B. RODMAN.*

From the Department of Pharmacology, University of Minnesota, Minneapolis, Minn., and the Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alta., Canada.

Wright, *et al.*,[1] have separated both arsphenamine and neoarsphenamine, by dialysis in an inert atmosphere, into a crystalloid and a colloid fraction. The crystalloid fraction is less toxic and more curative for trypanosomiasis in rats than either the whole drug or colloid fraction. Death from toxic doses of either the crystalloid fraction or whole drug was due to delayed arsenical poisoning. The colloid fraction produced acute toxic symptoms, such as extreme respiratory embarrassment and death from respiratory failure.

We have investigated the comparative distribution and retention of the whole drug and the crystalloid and colloid fractions of arsphenamine and neoarsphenamine in the tissues of the rat following their intravenous administration.

The drugs were injected intravenously in a dose of 15 mg/kg and the animals were sacrificed at intervals of ½, 2, 6, 12, 24, 168, and 336 hours, at which times blood, skin, muscle (skeletal), bone, liver, kidneys, spleen, brain, stomach, small intestine and colon were taken for analysis. The arsenic content was determined by the Gutzeit method of the A.O.A.C.[2] A total of 308 rats was employed and 2034 analyses of tissue arsenic were made. The results obtained with arsphenamine showed the following:

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* Accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Minnesota.

[1] Wright, H. N., Biederman, A., Hanssen, E., and Cooper, C. I., *J. Pharm. Exp. Therap.*, 1941, **73**, 12.

[2] Methods of Analysis, Official and Tentative, of the Association of Official Agricultural Chemists, 4th edition, 1935.

Blood. The amount of arsenic present in the blood stream in the case of the colloid fraction was at all times greater than that of the crystalloid fraction. This was particularly marked in the later time intervals.

Skin, Muscle, Bone, Spleen and Kidneys. With respect to the retention of arsenic in these tissues it was found that the retention of the colloid fraction was greater than that of the crystalloid fraction, both as to quantity and duration of retention. The crystalloid fraction showed a high initial penetration into these tissues, followed by a rapid lowering of the arsenic content. In contrast, the colloid fraction showed an appreciable concentration in these tissues at all time intervals. The whole drug showed retention of amounts of arsenic intermediate to those of the two fractions.

Liver. The liver showed a decided affinity for arsphenamine, 39.70% of the injected arsenic being found in this organ ½ hour after the injection of the whole drug, 35.05% of the crystalloid and 32.93% of the colloid fraction. The concentration of the crystalloid arsenic dropped more rapidly than either the whole drug or colloid fraction, both of which are long retained and eliminated slowly and incompletely over 14 days.

Brain. The amount of arsenic retained by the brain is small, only a fraction of a percent for any of the preparations studied. Somewhat greater penetration and retention was found for the whole drug and colloid fraction than for the crystalloid fraction.

Gastrointestinal Tract. The retention of arsenic in the stomach following the injection of these three drugs is small (approximately 1%) and little was found after 24 hours. The intestine, thoroughly washed free of contents, showed the high retention of 33.70%, 33.73%, and 29.85% for the whole drug, crystalloid and colloid fractions respectively at the ½-hour time interval. This had fallen to a small percent by the end of 24 hours. The colon retained only a small percent of arsenic with the exception of the 6-hour time interval at which time 7.40%, 13.69%, and 6.69% of the whole drug, crystalloid and colloid fractions respectively were recovered.

Total Recovery and Tissue Retention. The summation of the arsenic recoveries for all tissues shows that at the ½-hour time interval total recoveries of 108.7% for the crystalloid fraction, 116.2% for the colloid fraction and 109.2% for the whole drug were obtained. The amount of arsenic localized in the tissues, exclusive of blood, at the end of ½ hour, was found to be 90.82%, 89.46%, and

83.59% for the whole drug, crystalloid and colloid fractions respectively. Subsequent time intervals showed that the crystalloid fraction was eliminated more rapidly than the colloid fraction, which was long retained, the recoveries for the respective preparations at 2, 6, 12, and 24 hours being 49.81, 28.91, 7.43, and 6.06% for the crystalloid fraction and 53.79, 41.13, 28.79, and 33.45% for the colloid fraction. After 14 days less than 4% of the crystalloid fraction was recovered while in the case of the colloid fraction 36.92% was found to be still present in the body. The retention by the tissues of the whole drug was intermediate to that of the two fractions.

Neoarsphenamine. The results obtained in a similar study with neoarsphenamine were quantitatively different but identical in principle with those obtained for arsphenamine.

Conclusion. The separated colloid fraction of either arsphenamine or neoarsphenamine is retained long in the body in comparison with the crystalloid fraction which is rapidly eliminated.

13524 P

Prevention of Perosis by Biotin.*

Thomas H. Jukes and Francis H. Bird.
From the Division of Poultry Husbandry, University of California, Davis.

Perosis was reported[1] to be a symptom accompanying the egg white syndrome in chicks. In the present investigation, single-comb White Leghorn chicks were placed on the following diet at hatching: Yellow corn meal, 55 g; wheat middlings, 20; dried skim milk, 10; commercial casein, 10; ground limestone, 2; steamed bonemeal, 2; alfalfa meal, 1; choline chloride, 0.1; NaCl, 0.5; $MnSO_4$, 0.05; fresh raw egg white, 30 cc. The wet mixture was spread in thin layers and dried at 45°C, after which 0.3 g of fish oil blend (3000-A, 400-D) was added. In addition to dermatitis,[2] symptoms of perosis developed in from 50 to 70% of the chicks at the age of 3 to 5 weeks. If egg white was omitted from the diet, or if cooked egg white re-

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* Aided by a grant from the Williams-Waterman Fund of the Research Corporation, New York, N.Y.

[1] McElroy, L. W., and Jukes, T. H., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 296.

[2] Ringrose, A. T., Norris, L. C., and Heuser, G. F., *Poultry Sci.*, 1930-31, **10**, 166.

placed raw egg white, perosis did not develop. It was found in a preliminary experiment that all the symptoms were prevented by injecting a biotin concentrate[†] at a level which supplied approximately 3 μg of biotin daily. In the next experiment, crystalline biotin methyl ester[‡] was injected into the breast muscle. Each chick received a total of 13 μg divided into 7 injections during a period which extended from the 12th to the 32nd day. Eight chicks were used in the injected group and 11 uninjected chicks served as controls. The results shown in Table I were observed on the 38th day.

TABLE I.

Supplement	% of birds showing Perosis	 Dermatitis	Avg wt, g
None	55	82	216
Biotin	0	38	285

Feathering was superior in the injected group. The level of biotin administered, which averaged only 0.34 μg per bird per day for the duration of the experiment, was not sufficient to prevent dermatitis completely but was sufficient to prevent perosis.

13525 P

Lens Regeneration from the Iris in Urodeles and its Inhibition by Lens Reimplantation.*

FRANCES L. DINNEAN. (Introduced by L. S. Stone.)
From the Yale University School of Medicine.

In recent publications[1, 2] reviews have been given which point out that the best evidence of lens regeneration from the dorsal iris is limited to a small group of salamanders (Triturus). It is of interest to know whether or not all of the Triturus group possess this power of lens regeneration. Therefore the effect of lensectomy in *Triturus torosus* was investigated.

[†] Kindly supplied by Dr. Y. SubbaRow, Lederle Laboratories, Pearl River, N.Y.

[‡] Part of the biotin was generously furnished by Dr. Vincent du Vigneaud, and the remainder was purchased from the S. M. A. Company.

[*] Aided by grants to Dr. L. S. Stone from the John and Mary R. Markle Foundation and the Fluid Research Fund of Yale University School of Medicine.

[1] Stone, L. S., and Dinnean, F. L., *J. Exp. Zool.*, 1940, **83**, 95.

[2] Stone, L. S., and Sapir, P., *J. Exp. Zool.*, 1940, **85**, 71.

Stone and Sapir[2] reported that reimplanted lens tissue of the adult *Triturus viridescens* did not survive. Since the reimplant did not persist its inhibitory effect on lens regeneration could not be tested satisfactorily in their experiments. Therefore, in *Triturus torosus* the excised lens was reimplanted to test its capacity for survival. In case it did survive, any inhibitory effect on lens regeneration could be observed.

Lenses were completely removed in 100 eyes of *Triturus torosus* larvae ranging in length from 15 to 25 mm. For histological studies the animals were killed at close stages (2 to 50 days) after operation. The initial stages of lens regeneration were the thickening of the edge of the iris with subsequent depigmentation and separation of the cell layers. These three stages are distinct and progress at about the same rate of development. In all eyes fixed 5 days after operation the thickening of the dorsal iris was observed. At 7 days all specimens showed the first indication of slight depigmentation of the iris and from 9 to 14 days all eyes showed further depigmentation and separation of the cell layers of the dorsal iris. In cases fixed from 14 to 24 days after operation the regenerating lenses varied in developmental rate, especially in the formation of the fiber-forming pole. At 24 days 4 eyes had well-formed isolated lenses. Studies at frequent intervals from 27 to 50 days after operation revealed no new process of lens differentiation but merely one of growth of the regenerated lens.

In the second group of experiments lenses were removed and immediately reimplanted in 42 larval eyes of *Triturus torosus.* The animals were killed from 2 to 50 days after operation. Nineteen of the 42 eyes had reimplanted lenses which appeared normal. These were found in specimens fixed as late as 50 days after operation. In these eyes the dorsal margin of the iris showed no changes that could be interpreted as stages of lens regeneration.

Eleven of the 42 eyes had reimplanted lenses which were cystic. The vacuolated area in 2 extreme cases equaled about one-half the total volume of the lens. In spite of the fact that lenses in this group had areas of degeneration, the dorsal rim of the iris in these eyes showed as in the other group no indications of an attempt to regenerate a lens.

In the remaining 12 eyes of this group the reimplanted lenses were either absent or present as small remnants of lens tissue. Three of these eyes were fixed within a week after operation, in one of which the corneal wound had not closed. Since the removal of the intact lens required a large corneal slit, the absence of these lenses and possibly others in this group may have been due to the early escape of lens tissue before the corneal wound had finally closed.

In the other 9 eyes in which no lenses were found the specimens were fixed later than one week after operation. Therefore, how long the implanted lens tissue remained in these eyes it is impossible to state exactly. However, the appearance of the dorsal iris may give some clue. For example, in a 15-day case the reaction of the dorsal iris was comparable to that of a 7-day lensectomized eye, indicating that the onset of lens regeneration had been retarded. This delay might have been due to the temporary presence of reimplanted lens tissue. More detailed studies are planned to test further this possibility.

13526

Studies of the Action of Sulfonamides upon the Bacterial Cell.

W. HARRY FEINSTONE, ROGER D. WILLIAMS AND H. J. FLORESTANO. (Introduced by E. K. Marshall, Jr.)

From the Research Laboratories, American Cyanamid Company, Stamford, Conn.

The mode of action of sulfonamides upon susceptible bacterial species has been the subject of much study. It appeared to us that one of the most fundamental aspects of this problem was to determine whether active compounds differ from closely related inactive substances in their respective abilities to reach or penetrate the bacterial cell. It was conceivable that p-aminobenzene sulfonamide (sulfanilamide) diffuses into the streptococcal cell (and is concerned in its metabolism) whereas its chemotherapeutically inert isomers, the ortho and meta compounds, do not diffuse into the organism. Since this does not appear to be true, the inactivity of certain compounds cannot be explained by this one fundamental property. In the light of our results diffusion into the organism may or may not be a requisite for a chemotherapeutic agent.

Twenty-liter batches of broth cultures of *Streptococcus hemolyticus,* strain C-203, were grown for 48 hours. The organisms were then collected by means of a continuous flow Sharples centrifuge and washed with 250 cc normal saline 6 to 8 times. The cells were collected after each washing by means of horizontal centrifugation. After final washing the bacteria were dried over P_2O_5 in a vacuum desiccator. The same procedure was repeated with cultures in broth containing 20 mg % sulfanilamide, orthanilamide and metanilamide, respectively. The drug broth cultured bacteria were washed 6 to 8 times until the wash fluid contained no demonstrable sulfonamide compound as determined by the Marshall test. Accurately weighed samples of dried organisms from each of these test cultures were quantitatively extracted in 3% trichloracetic acid for 2 hours at 37°C and the amount of each substance so eluted was also determined by the Marshall method. These experiments were performed twice with each of the 3 compounds and several samples from each experiment were subjected to analysis.

Table I shows that 4 to 12 mg of drug per 100 g dry weight of bacteria were extracted from each of the samples regardless of which of the isomers was used except that there was a rough correlation with solubilities of the respective compounds. This may be taken to indicate that the drugs either penetrate the cell or are strongly

TABLE I.
Concentrations of Sulfanilamide and Its Isomers in Washed Streptococci Grown in Broth Containing These Drugs.
Conc. in mg per 100 g Dry Wt of Streptococci.

Control	Sulfanilamide	Orthanilamide	Metanilamide
0 (blank 1.0)	5.0-9.0	4.0-7.0	7.0-9.0
0 ”	5.0-9.0	4.0-7.0	7.0-12.0

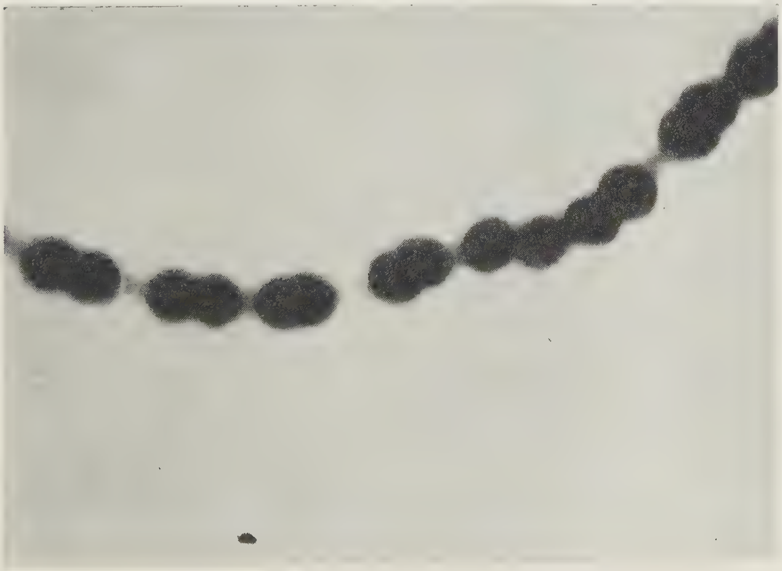

PLATE 1.
Streptococcus hemolyticus, Strain C-203, 24-hour culture grown in plain beef infusion broth. Cells diluted with saline.

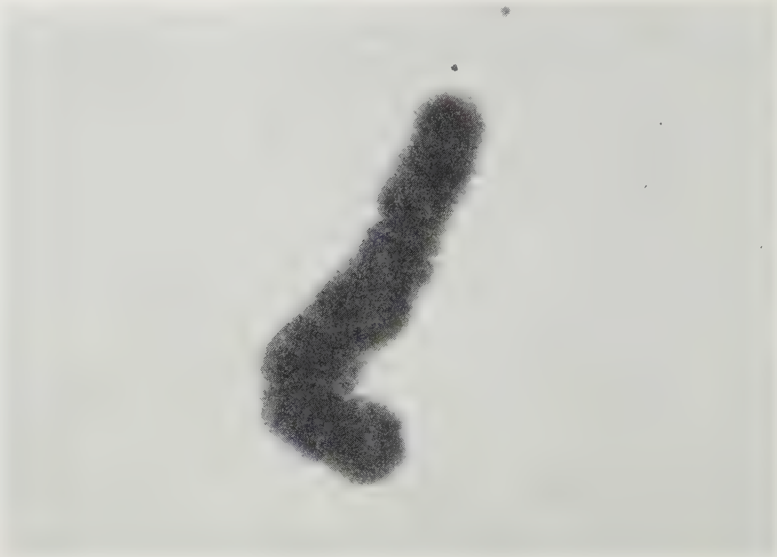

PLATE 2.

Streptococcus hemolyticus, Strain C-203, 24-hour culture grown in broth containing 10 mg % sulfanilamide. Cells diluted with saline.

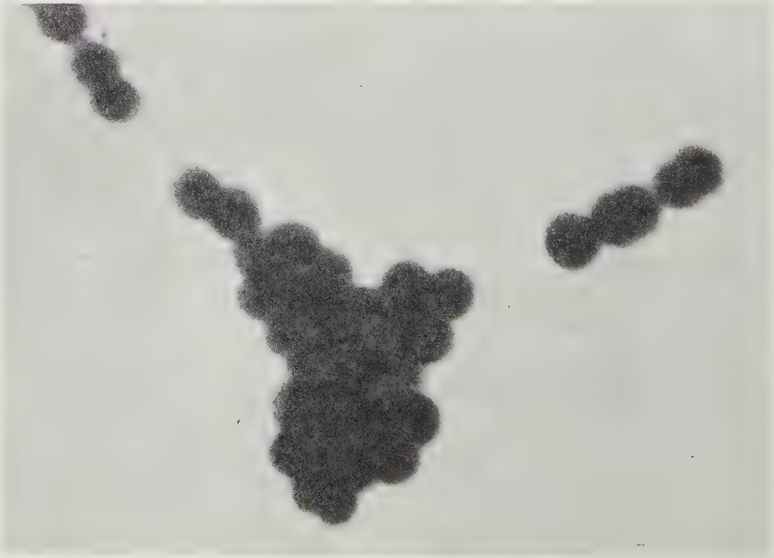

PLATE 3.

Streptococcus hemolyticus, Strain C-203, 24-hour culture grown in broth containing 10 mg % sulfanilamide. Cells washed in saline 3 times.

adsorbed upon the surface so that 6 to 8 washings do not remove them; also that there is no difference in this between the active sulfanilamide and its inactive ortho and meta isomers. However, this result gives no indication whether this localization is due to adsorption or diffusion of all the compounds or perhaps diffusion in the case of sulfanilamide and adsorption in the case of the inactive ortho and meta isomers.

It was felt that the electron microscope* might offer valuable aid

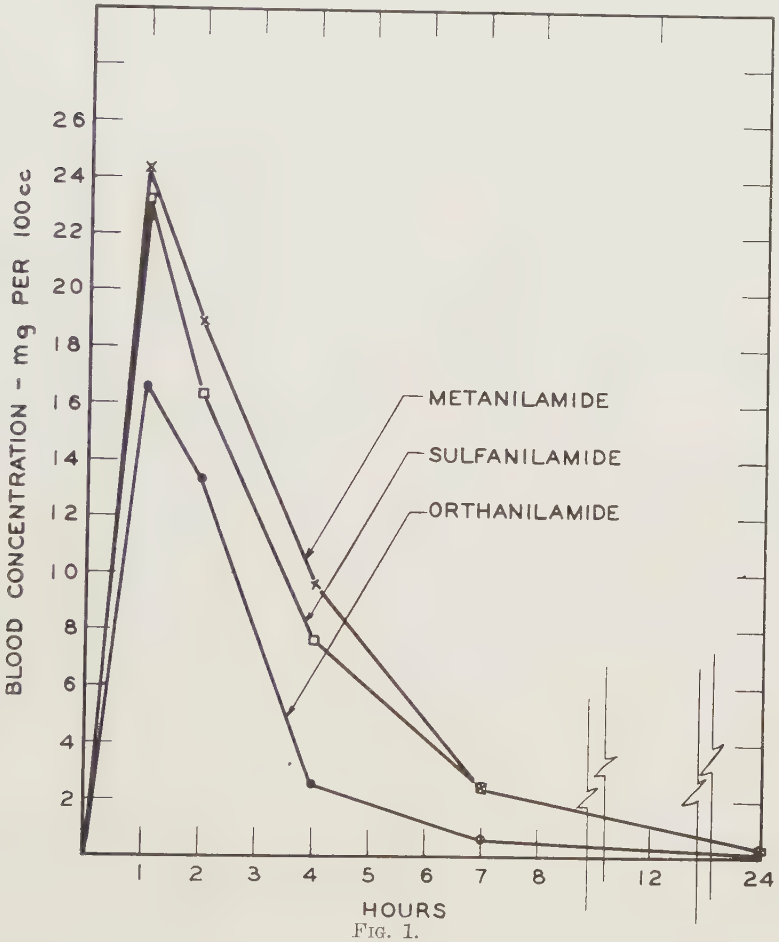

FIG. 1.

Blood concentration-time curves in mice following 10 mg oral doses of sulfanilamide, orthanilamide and metanilamide.†

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* We are grateful to Mr. Charles Burton of these laboratories for the electron photomicrographs.

† Quantitatively pooled blood from 10 mice for each curve. Color readings made on Hardy spectrophotometer. Orthanilamide and metanilamide presented difficulty in obtaining standard curves. Maximum color formation for diazotized and coupled orthanilamide required 30 minutes. Metanilamide in about 12 experiments never yielded a constant standard curve. The results shown here for metanilamide are therefore only approximate but not appreciably in error.

in answering this question. Numerous photographs of streptococci growing in plain broth showed the normal appearance of the organism by this method to be quite constant (Plate 1). The examination of cells grown in broth containing 10 mg % of either sulfanilamide or its isomers and washed only once or not at all showed characteristic crystals surrounding the organisms (Plate 2). However, when the organisms were washed 2 or 3 times, the appearance of the streptococci was similar to normal organisms, the crystals having been washed away (Plate 3). Referring to the first experiments, this evidence insofar as it may apply suggests that the drugs diffuse into the organism rather than that they are adsorbed onto the surface of the bacterial cell.

Figure 1 shows the blood concentration time curves of sulfanilamide and its ortho and meta isomers as obtained in mice following oral administration of 10 mg of the respective compounds. All of these substances are well absorbed from the gastrointestinal tract and are therefore available for action *in vivo*.

In vivo and *in vitro* studies on the ortho and meta isomers prove them to be completely inert as chemotherapeutic agents in streptococcal and pneumococcal infections whereas the activity of the para isomer, sulfanilamide, is well known. It must be concluded, therefore, that availability to the host by absorption and distribution, and to the bacteria by strong adsorption or diffusion through the cell, is not a criterion of chemotherapeutic activity of any given compound. Inactive substances may manifest the properties of availability to the host and diffusion into or adsorption onto the bacterial cell but do not inhibit reproduction or otherwise destroy the organism by participating in the metabolism, in the manner that has been suggested for active substances such as sulfanilamide.

13527

Preservation of *Bartonella muris* in the Frozen State.

WALTER R. KESSLER. (Introduced by A. R. Dochez.)

From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York.

Bartonella muris has been cultivated only with difficulty and but few workers have reported success.[1, 2, 3] All used Noguchi's leptospira medium or some minor modification of it. Recently two new methods for the cultivation of the human bartonella, *Bartonella bacilliformis,* have been described. One[4] involves the use of the chorioallantoic membrane of the fertile hen's egg; the other does not require living tissue. Geiman[5] reports good growth of *Bartonella bacilliformis* on a serum-tryptone medium to which small amounts of ascorbic acid and glutathione are added. Whether these media are suitable for the growth of *Bartonella muris* of the rat has not yet been demonstrated. Preliminary attempts by the author to cultivate *Bartonella muris* by inoculating infected blood directly into the yolk sac of the fertile hen's egg, have as yet yielded negative results.

In view of the difficulty involved and the meager success obtained in cultivating *Bartonella muris,* a simple method of preserving this infective agent over long periods is desirable. Alsted,[6] when testing the effect of storing *Bartonella muris* at various temperatures, noted no change in infectivity after the preservation of infective blood at 0°C for 24 hours. Weiman[7] reported success in maintaining the infectivity of a related species, *Eperythrozoon coccoides,* for 5 days when infective blood was stored at 5°C. However, when tested after storage for 10 days the material was no longer infective. It occurred to the author that if the organisms were stored at extremely low temperature they might survive storage for prolonged periods. This method of storage is not new, having been used successfully by Turner[8] for the preservation of the spirochetes, *Treponema pallidum* and *Treponema pertenue,* as well as by Coggeshall[9] for the malarial organisms, *Plasmodium knowlesi* and *Plasmodium inui.*

Method. By means of cardiac puncture, blood was drawn from a series of splenectomized rats at the peak of their infection with *Bartonella muris,* when a large percentage of their erythrocytes showed the presence of the parasite. The blood was defibrinated and pooled. After a portion of the blood was set aside to serve as a control upon the infectivity of the fresh blood, 0.5 cc samples were

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[1] Noguchi, H., *J. Exp. Med.,* 1928, **47**, 235.

[2] Marmorston-Gottesman, J., and Perla, D., *J. Exp. Med.,* 1932, **56**, 763.

[3] Lawkowicz, W., *Trans. Roy. Soc. Trop. Med. and Hyg.,* 1939, **32**, 601.

[4] Jiminez, J. F., and Buddingh, G. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 546.

[5] Geiman, Q. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 329.

[6] Alsted, G., *Acta. Path. et Microbiol. Scandinav.,* 1938, **37**, 37.

[7] Weiman, D., *Les Parasites Érythcytaires Révélés par la Splénectomie: Bartonella et Epérythrozoon,* 1935, Paris, Amédée Legrand.

[8] Turner, T. B., *J. Exp. Med.,* 1938, **67**, 61.

[9] Coggeshall, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 499.

placed in Wassermann tubes and sealed with rubber stoppers. The tubes were immersed in a freezing mixture of dry ice and alcohol and rotated rapidly to insure uniform and rapid freezing of the infected blood. They were then packed in dry ice and stored in a thermos jug of one gallon capacity, the dry ice being replenished as required. The frozen blood was tested for infectivity at various intervals thereafter. Immediately before use the blood was liquefied by immersing the tubes in warm water (40-45°C). Melting occurred rapidly. Shortly thereafter, 0.1 cc samples were inoculated intraperitoneally into splenectomized bartonella-free rats. The test animals were all from 45 to 50 days of age and had been splenectomized immediately before infection.

Results. Six rats were injected with the control, fresh blood (Series I). Two days later Bartonella-infected red cells were present in Giemsa stained films of the tail blood of all the animals. Five of the rats succumbed on the fourth day and one on the sixth day.

The frozen blood was tested after storage for 3, 5, 9, and 11 weeks. After storage for 3 weeks (Series II) there was no signifi-

TABLE I.
Infectivity of *Bartonella muris* Preserved in the Frozen State.

Series No.	No. wks blood stored	Rat No.	Days after infection					
			1	2	3	4	5	6
I	0	1	0	+	++	D		
	0	2	0	+	++	D		
	0	3	0	+	++	D		
	0	4	0	+	++	D		
	0	5	0	+	++	D		
	0	6	0	+	+	++	++	D
II	3	7	0	0	+	D		
	3	8	0	0	+	D		
	3	9	0	+	++	D		
III	5	10	0	0	+	D		
	5	11	0	0	++	D		
	5	12	0	0	+	++	D	
	5	13	0	0	+	++	++	D
IV	9	14	0	0	0	+	D	
	9	15	0	0	0	+	D	
	9	16	0	0	0	+	D	
V	11	17	0	0	0	+	++	D
	11	18	0	0	0	+	++	D
	11	19	0	0	0	+	++	D

All rats infected with 0.1 cc of blood.
0 = no cells infected.
+ = less than 50% of red corpuscles infected.
++ = more ” 50% ” ” ” ”
D = dead.

cant change in the infectivity of the frozen material. Two of the 3 rats injected with this material showed a prepatent period of 3 days. Yet, all the animals died on the fourth day. Two of the 4 animals injected with blood stored for 5 weeks (Series III), died on the fourth day after infection; the others succumbed on the fifth and sixth days respectively. Animals infected with blood stored for nine weeks (Series IV), showed a further increase in the prepatent period to four days. However, there was little or no loss in the virulence of the parasite, for all the animals succumbed on the fifth day. Rats injected with blood stored for 11 weeks (Series V) showed the same prepatent period as those given blood stored 9 weeks, but death occurred on the sixth day.

At the time the last series was tested the virulence of the parasites in the frozen blood had not changed, using the death of the infected animals as the criterion. The gradual increase in the length of the prepatent period of the infected animals, which resulted from longer storage, was probably due to the destruction of some of the Bartonella with preservation. The infective dose thus representing fewer infective units than the original stock, although these were of undiminished virulence. Doubtless the 11-week period during which the blood was stored by no means indicated the maximum length of time during which *Bartonella muris* may be preserved in the frozen state.

Summary. The infectivity of *Bartonella muris* can be preserved for at least 11 weeks when infected defibrinated rat blood is rapidly frozen in a mixture of dry ice and alcohol and then preserved in dry ice.

The author wishes to express his gratitude to Dr. J. T. Culbertson for his constant help, to Mr. F. L. Rights for demonstrating the procedure used in freezing and storing the parasite, and to Miss E. Molloy for her aid in our attempts at cultivation.

13528

Poliomyelitis Mouse Neutralization Test, Applied to Acute and Convalescent Sera.*

WM. McD. HAMMON AND E. M. IZUMI. (Introduced by K. F. Meyer.)

From the George Williams Hooper Foundation, University of California, San Francisco, California.

Through the adaptation of a strain of poliomyelitis virus to the mouse by Armstrong,[1] a mouse neutralization test has been made possible. Haas and Armstrong[2] have already applied this test to post-epidemic surveys, but with results which are difficult to interpret. Thus, we felt it wise to investigate its significance somewhat further by testing a series of acute and convalescent sera from poliomyelitis patients against this same virus. Twenty-three pairs of such sera have now been tested. They were collected from 3 different outbreaks over a period of 2 years. Sera from 1940 and 1941 from the Los Angeles County Hospital were supplied by Dr. John F. Kessel. Another group, collected during the Tacoma, Washington, epidemic in 1940, were obtained from Miss Beatrice Howitt. None of the patients were seen by us, but the clinical diagnoses were made and checked by clinicians well trained in infectious diseases. The diagnosis of the non-paralytic case, in particular, is liable to error, but spinal fluid examination in all instances, together with serum neutralization tests to eliminate the virus encephalitides in several instances, reduced the possibility of error. From one non-paralytic case poliomyelitis virus was isolated from the stool by Miss Howitt.

Method. Both the admission (acute) and the discharge (convalescent) serum from each patient were tested simultaneously to insure comparable results. Positive and negative controls were always tested at the same time. A minimum of 8 mice was used for each serum, and tests on many pairs were repeated to check results. The first series was run by the method described by Armstrong,[2] employing 3 twofold dilutions of virus, but later a technic was adopted which is more comparable to that used for the yellow fever virus, using a large number of mice and only one dilution. A stock, frozen (–76°C), virus was used in all tests. The 50% end point of this frozen virus was calculated by the method of Reed

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Armstrong, Charles, *Pub. Health Rep.*, 1939, **54**, 2302.

[2] Haas, V. H., and Armstrong, C., *Pub. Health Rep.*, 1940, **55**, 1061.

and Muench after titration under the exact conditions of the neutralization test, each dilution of virus mixed in equal parts with a known negative serum. A dilution containing 10 times the virus of the 50% end point was used for the neutralization test. We found that this selected dilution contained approximately 100 times the greatest quantity of virus which would just permit survival of all mice when mixed with a known negative serum. A few human sera have been found capable of protecting all mice in the presence of a greater amount of virus than that selected, but this dilution was found to represent the approximate limit of protection of the average adult serum. A serum able to protect half the mice was interpreted as a weak positive (+), and one protecting all or all but one mouse as a strong positive (++). All mice were observed for 21 days for paralysis or death. Further details regarding the method and our reasons for adopting it have been discussed elsewhere.[3] Over 200 sera have now been tested against the mouse-adapted poliomyelitis virus by this technic, and we are satisfied as to the constancy of results in our hands.

TABLE I.
Neutralization Tests on Acute and Convalescent Sera of Poliomyelitis Patients.

Case	City	Year	Age	Days interval between bleedings	Days after onset final bleeding	Type of disease	Acute serum	Convalescent serum
1	Los Angeles	'40	4	16	22	Spinal paralytic	0	0
2	" "	"	9	8	24	" "	0	0
3	" "	"	7	19	21	" "	0	+
4	" "	"	14	18	20	Non-paralytic	0	+
5	Tacoma	"	7	10	16	Bulbar paralytic	0	++
6	"	"	9	20	21	" "	0	++
7	Los Angeles	'41	11	—	21	Spinal "	0	++
8	" "	'40	15	13	16	Non-paralytic	0	++
9	Tacoma	"	15	27	29	Bulbar paralytic	0	++
10	Los Angeles	"	16	14	17	Spinal "	0	++
11	" "	'41	30	—	22	" "	0	++
12	" "	"	12	—	21	Non-paralytic	+	++
13	Tacoma	'40	9	14	19	" "	++	++
*14	"	"	12	17	22	Bulbar paralytic	++	++
15	Los Angeles	'41	13	—	20	" "	++	++
16	Tacoma	'40	15	15	17	Non-paralytic	++	++
17	"	"	18	14	17	" "	++	++
18	Los Angeles	'41	18	—	31	Spinal paralytic	++	++
19	Tacoma	'40	25	16	20	Non-paralytic	++	++
20	Los Angeles	"	28	16	21	Spinal paralytic	++	++
21	" "	"	28	18	21	Bulbar and spinal paralytic	++	++
22	" "	"	30	19	21	Non-paralytic	++	++
23	" "	"	38	20	21	" "	++	++

*Poliomyelitis virus isolated from feces.

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[3] Hammon, W. McD., and Izumi, E. M., *J. Immunol.*, in press.

Findings. In Table I are presented the results of the tests on the 23 pairs of acute and convalescent sera. The patients' ages ranged from 4 to 38 years with a mean of 17.1 and a median of 15. This group was obviously above the average in age for this disease.

Acute bloods from 11 patients showed no protection. Two of these failed to develop definitely demonstrable antibody 22 and 24 days after onset. These were both spinal paralytic cases. Nine of the 11 negatives changed to positive in from 16 to 29 days after onset. Two of these were non-paralytic, 2 were bulbar and 5 spinal paralytic. One non-paralytic patient's serum changed from weakly positive to strongly positive 21 days after the onset.

A group of 11, composed principally of non-paralytic cases (6 non-paralytic, 1 bulbar and 3 spinal paralytic), showed strong protection at the time of both bleedings.

Discussion. Considering the limited time of hospital observation, 16 to 29 days, it appears quite significant that 9 of 11 patients whose serum was originally without antibody showed a definite increase during the course of the disease, a large increase in most instances. Another, changing from a weak to a strong positive increases the series responding with a definite increase of titer to 10 out of 12.

The series of 11 possessing a high titer of antibody at the time of first bleeding raises a question not possible to answer at this time. It will be noted, however, that this group has a higher mean and median age than does the preceding series; also contains more non-paralytic cases. This antibody, associated with relative immunity, was most likely due to previous inapparent infection and accounts in part, at least, for the infrequent attacks of recognized infection in this older age group. This resistance is probably, however, only relative and may be broken down by some factor such as overwhelming dosage. As suggested by recently published data, immunological differences between strains of poliomyelitis viruses might also be considered in attempting to explain clinical poliomyelitis in persons already endowed with serum neutralizing antibody to one strain of virus.

Larger series of paired sera from many geographical areas should help to answer some of the problems. However, unless a standardized method is employed for both the test and its interpretation, a comparison of various series may very well result in confusion rather than clarification.

The practical application of the mouse neutralization test as a diagnostic laboratory method is obviously limited. In the first place, an acute stage serum must be available to be tested with the convalescent specimen. Also, if the first specimen is strongly positive (in this very limited series about 50% of the cases) the test would appear to offer no help in diagnosis. Case 14, in which the diagnosis for this reason could not be confirmed by the neutralization test, yielded virus by inoculation of a stool specimen.

As to the application of this test to population surveys, it seems probable that it will serve in the same way as has the monkey test, and possesses no advantages other than those resulting from economy.

13529

Physiological Observations Upon Larval Eustrongylides. III. Culture Attempts *in vitro* Under Sterile Conditions.

THEODOR VON BRAND AND WILLIAM F. SIMPSON.

From the Department of Biology, The Catholic University of America, Washington, D.C.

Many phases of the metabolism of internal parasites can be studied only with animals kept *in vitro*. Most helminths, however, live only a short time outside their host. A serious objection[1, 2] against such investigations is that the data may be derived from dying animals and may not be representative for healthy specimens. Preliminary experiments mentioned in the first paper of this series[3] showed that a larval Eustrongylides could be kept alive for long periods in sterile surroundings. The goal of the present investigation was to find a medium that would favor the survival *in vitro* of this worm and be simple enough to promise success for future chemical investigations.

Material and Methods. The worms used were larval Eustrongylides (probably *Eustrongylides ignotus*) from *Fundulus heteroclitus*. The sterile extraction of the nematodes required 2 persons. The fish was killed by pithing. It was pinned down on its back and its entire ventral surface was painted with a strong alcoholic iodine solution. The abdominal cavity was opened with sterile instruments and the worm cysts located. Their surface was sterilized by application of iodine solution, and the cyst was then torn open with two

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[1] Lapage, G., *Nematodes Parasitic in Animals*, Chemical Publishing Co. of N. Y., Inc., 1938, 30.

[2] Stunkard, H. W., *J. Parasitol.*, 1940, **26**, 1.

[3] von Brand, Th., *J. Parasitol.*, 1938, **24**, 445.

sterile forceps. The worm was seized with one of the forceps and dropped immediately into the test-tube containing the sterile medium which was handled with the usual aseptic precautions by the second person. Varying in the different series, from 80 to 100% of the isolated worms remained sterile, as evidenced by the fact that the medium remained entirely clear despite incubation periods of several weeks at 37°C. In over 100 cases test-tubes with sterile sugar-broth were inoculated with medium from tubes in which worms had been kept for about 4 weeks. The broth remained clear in all cases in which the worms previously had been classified as sterile.

All media, except heat labile substances were sterilized by autoclaving at 15 lb pressure for 20 minutes. Blood serum and Tyrode's solution were passed through Berkefeld "N" filters.

The worms were kept singly in ¾-inch by 6-inch test-tubes containing 12 cc medium. They were closed with a cotton plug and parafilm, thus effectively preventing evaporation, and kept at 37.5 ± 1°C. At intervals of 4 to 8 weeks the old solution was replaced by a similar amount of freshly prepared medium. The pH of the media were measured by means of a potentiometer both at the beginning and the end of the period.

The viability of the nematodes was controlled 2 or 3 times a week during the first weeks after isolation, later on, once or twice a week. The worms remained usually curled up at the bottom of the test-tube, but showed throughout their life distinct movements, especially if subjected to strong light. A few days before death they usually extended in a few large loops through the whole medium. A nematode was considered dead when it had lost its bright red color or when it did not react with movements to light.

Results and Discussion. A summary of our results is presented in Table I. It is apparent that as basic medium Bacto broth, Bacto yeast extract, Proteose peptone and peptone were about equally well suited. The addition of between 0.5 and 1% NaCl was highly beneficial, and proved to be more effective than the use of Tyrode solution. The concentration of the organic material, too, could be varied widely; in the case of broth the optimal limits were about 0.8 to 1.6%. Dextrose in a concentration of 0.5% more than doubled the average length of life both in broth and yeast extract.

The addition of liver extract or serum, the use of agar-slants of various types covered with different fluids did not improve the survival; on the contrary, it was in general shorter.

The pH of the solutions dropped invariably during the incubation period. It can be assumed that the actual production of acids was

TABLE I.
Survival of a Larval Eustrongylides in Various Sterile Media.

Medium	No. of sterile worms	Length of life in days			pH	
		Avg	Min.	Max.	Initial	Change after 4-8 wks
Bacto-Yeast extr. .5%	5	5	4	6	6.78	?
” ” ” ” + NaCl .5%	5	72	22	97	6.67	— .21
” ” ” ” ” ” ” + glucose .5%	6	152	67	346	7.00	— .61
Proteose-Peptone 1% + NaCl .5% + glucose .5%	9	129	98	245	7.23	— .47
” ” ” in Tyrode + ” ”	7	82	38	240	7.80	— .31
Rabbit serum 1% in Tyrode + glucose .5%	17	43	13	94	8.50	— .54
Ascites fluid + glucose .5%	6	38	4	65	7.65	— .63
Endamoeba liver agar slants covered with broth .8% + NaCl .85% + glucose .5%	17	20	3	49	7.10*	?
Endamoeba liver agar slants covered with Tyrode + glucose .5% + serum 1%	11	23	3	61	8.50*	?
Agar slants (agar 2% + glucose .5% + NaCl .5% covered with yeast extr. .5% + NaCl .5% + glucose .5%)	18	123	70	248	7.13*	—1.51
Peptone 1% + NaCl .85% + glucose .5%	18	105	5	298	7.36	— .99
Bacto-broth .8% + NaCl .5%	5	64	10	105	6.70	— .10
” ” ” ” ” + glucose .5%	8	157	103	228	6.93	— .24
” ” ” ” 1% ” ” ”	10	114	56	200	7.17	— .65
” ” ” ” 3% ” ” ”	12	11	2	16	7.04	?
” .2% ” ” .85% ” ” ”	13	116	45	195	6.91	—1.35
” .4% ” ” ” ” ” ”	11	92	21	226	7.03	—1.04
” 1.6% ” ” ” ” ” ”	19	148	83	284	7.17	— .56
” 2.5% ” ” ” ” ” ”	17	68	33	93	6.82	— .40
” .8% ” ” .5% ” ” ” + Lederle's liver extr. 1%	9	77	17	121	7.07	— .63
Bacto-broth .8% + NaCl 3% + glucose .5% + Lederle's liver extr. 2%	12	12	2	14	6.88	?
Bacto-broth .8% + NaCl .5% + glucose .5% + horse serum 12%	9	15	6	72	6.39	?
Bacto-broth .8% in Tyrode + glucose .5%	14	90	30	163	7.85	— .62

*Figures represent the pH of the supernatant.

larger than indicated by the pH changes, since the materials present in the media have a considerable buffer capacity. This is indicated by a comparison of the average pH drop of 0.40 in the medium containing 2.5% broth with that of 1.35 in the medium containing 0.2% broth.

The average and maximal survival periods in the best media were considerably longer than those hitherto reported for Nematode larvae kept at body temperature (older literature in Hoeppli, Feng and Chu,[4] new data for *Haemonchus larvae* Stoll.[5] *Trichinella larvae* Lewin[6]). No definite sign of growth or development was seen, in this respect we were less successful than Stoll, but the worms did certainly not become smaller, a phenomenon so apparent even at much lower temperature in other worms (Planaria) kept starving. The worms must either have been able to utilize the foodstuffs contained in the media, or the rate of their metabolism must have been exceedingly lowered. Previously published data[3] indicate that the carbohydrate reserves of the worms would last only for 23 days if the rate of metabolism remained unchanged. The observation that sugar in the medium increased the length of life *in vitro* might point towards its actual utilization. At the present time we are inclined to assume that our results approach those achieved with several pathogenic trypanosomes which in suitable media thrive in the stages characteristic for the intermediate host but do not change into the blood forms.

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[4] Hoeppli, R., Feng, L. C., and Chu, H. J., *Chinese Med. J.*, 1938, Suppl., **2**, 385.

[5] Stoll, N., *Growth*, 1940, **4**, 383.

[6] Lewin, A. J., *J. Parasitol.*, 1940, **26**, Suppl., 31.

Summary. 1. The maximal survival *in vitro* of an individual Eustrongylides larva at 37.5°C was 346 days. The medium was Bacto Yeast extract 0.5% + NaCl 0.5% + Glucose 0.5%. 2. The maximal average survival of a series of worms was 157 days in a medium containing Bacto-broth 0.8% + NaCl 0.5% + Glucose 0.5%. 3. The molecular concentration of the media could be varied in rather wide limits without material change in the length of survival. 4. The presence of 0.5% glucose was decidedly beneficial. 5. The worms produced acids. 6. No moulting or other sign of development was observed.

13530 P

Ascorbic Acid Requirement of Individuals in a Large Institution.

M. K. HORWITT.

From the Biochemical Research Laboratory, Elgin State Hospital, Elgin, Illinois.

A three-year accumulation of data obtained from the analysis of more than 1,000 blood samples indicated that the average plasma ascorbic acid level of the patients in this hospital was less than .40 mg %. The lowest results, averaging .20 mg %, were obtained during the months of April, May and June and the highest during October and November when an average of .59 mg % was achieved. These data on ascorbic acid and others on various nutritional essentials which will be described in subsequent reports indicated that a large mental institution is an exceptional laboratory for conducting studies of human nutrition and a project has been started to make use of this opportunity. The regulated routine of the majority of the patients, the supervision of the dietary intake, and the choice of age and sex from the large numbers available make it possible to have better controls than is ordinarily so easily available.

A modification of the Mindlin and Butler[1] method was used to determine the plasma ascorbic acid. No cyanide was added to the blood but the plasma was separated from the cells and precipitated with metaphosphoric acid within 30 minutes after the blood was drawn in order to minimize ascorbic acid oxidation. All recent estimations have been made with the aid of a Cenco Spectrophotometer. Prior to January, 1941, a Cenco Photelometer was used with a green filter. All blood samples were taken in the morning before breakfast.

Table I presents the average ascorbic acid levels of different groups who were in residence at this hospital during the months March through June. The differences demonstrated are the direct results of differences in the diets. The patients who had the freedom of the grounds purchased fresh fruits at the commissary; the infirmary patients had been given fruit juices as a regular routine for several years; the dining-room attendants are patients who eat essentially the same diet as the hospital staff, and the staff supplemented their regular meals by the consumption of fresh fruit in their quarters.

In order to determine the minimum amount of ascorbic acid which should be added to the diet to remedy this apparent deficiency in the average patient, an experiment was conducted as follows: A ward containing 40 young, male, schizophrenics was divided into 2 groups of 20 patients each. Starting on April 15, 1941, 50 mg of crystalline ascorbic acid, made up in capsules, were given to Group I twice a week for 2½ weeks. Analyses of blood samples drawn before breakfast, about 20 hours after the ascorbic acid had been taken, indicated that 50 mg given twice a week did not bring about any significant change in the plasma ascorbic acid. When the supplement was increased to 50 mg every other day, there was a gradual in-

TABLE I.
Average Ascorbic Acid Levels During March-June (mg%).

Average patient (380)	.20
Patients with ground passes (76)	.38
Infirmary patients (40)	.56
Patients in staff dining rooms (54)	.80
Staff (30)	1.0

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[1] Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

TABLE II.

Average Ascorbic Acid During Experiment to Determine Amount of Supplement Necessary.

Twenty patients in each group. Samples taken before breakfast approximately 20 hours after administration of ascorbic acid.

Group I received 50 mg twice a week 4/15 to 5/8 and 50 mg every other day 5/9 to 7/1.

Group II served as control (no ascorbic acid).

Date	4/15	5/9	5/23	6/2	6/16	7/2	7/16
Group I	.20	.21	.34	.43	.59	.74	.52
Group II	.25	.14	.19	—	.15	.17	—

crease in the ascorbic acid content of the blood, as shown in Table II, and after 6 weeks on this regime a more satisfactory value (.74 mg %) was obtained. Withdrawal of the ascorbic acid supplement from Group I caused a decrease in the average plasma ascorbic acid of .22 mg % in 2 weeks. It is interesting that this decrease almost parallels the size of the increase during the 2-week intervals before the ascorbic acid was withdrawn. The second group, which served as the control and received no ascorbic acid, remained at a constant low level throughout the experiment.

A dietary calculation of the ascorbic acid in the food served to the patients during the time of this experiment showed that there was not more than 25 mg in the average daily diet. Since the addition to this diet of 50 mg every 48 hours was sufficient to bring about a slow but steady increase of the plasma ascorbic acid until the blood level was higher than .7 mg %, it can be stated that 50 mg per day is sufficient to keep the sedentary individual in a positive ascorbic acid balance.

The practical problem of supplying vitamin C to individuals fed from kitchens that produce several thousand meals a day on a limited budget is quite different from that encountered in ordinary nutritional practice. While the addition of purified vitamins to the diet should always be considered as an inferior substitute to the use of the proper foods, there are times when the proper foods are not so easily available and the addition of the purified vitamin is justifiable. In the case of ascorbic acid the synthetic substitute is considerably less expensive than the fresh or canned food—approximately 1 cent per week per patient—and it is a simple matter to add ascorbic acid to some of the food after it is cooked.

The author wishes to express his appreciation to Dr. Charles F. Read for his interest and support of this investigation.

13531 P

Loss of Prothrombin Activity in Plasma Exposed to Air Currents.

L. M. Tocantins.

From the Division of Hematology, Department of Medicine, Jefferson Medical College, Philadelphia, Pa.

During the early summer of 1941 it was noticed that oxalated plasma collected for the quantitative determination of prothrombin by the Quick method[1] underwent a marked decrease in activity after standing a few hours, uncovered, directly in the cool draft of a York air-conditioning unit. The experiments reported indicate that this decrease in prothrombin activity is due to rapid evaporation and loss of CO_2 by the plasma. Addition of CO_2 even by the simple method of saturating the plasma with expired air restores prothrombin activity to its initial level.

1. Changes in prothrombin time of plasma exposed at 38°C for 1 hour to a continuous draft from an air conditioning machine at a distance of 8 feet.

Another specimen of plasma was placed in a 15 cc centrifuge tube, uncorked, 30 cm in front of a fan. The prothrombin time before exposure was 16.8 sec. After 2 hours of exposure it was 53.7 sec. A 1-1 dilution of this plasma with .85% sodium chloride gave a prothrombin time of 130.9 sec. Exposure of plasmas at room temperature to still air for 4 hours results in only a slight decrease in prothrombin activity. Analogous results have been obtained with oxalated or citrated rabbit and dog plasma.

2. Effect of exposure to oxygen or CO_2 on the prothrombin activity of fresh plasma. Plasma was separated under oil from oxalated dog blood, placed in a 15 cc centrifuge tube and oxygen

TABLE I.

July 20, 1941. Plasmas separated from human oxalated blood (0.1 vol. of 1.34% sodium oxalate solution added to the blood). 0.1 cc plasma, 0.1 cc rabbit brain thromboplastin, 0.1 cc 0.025 M $CaCl_2$.

	Prothrombin time (sec) at		
Specimen No.	4.45 p.m.	5.20 p.m.	6.00 p.m.
1	17.6	20.4	21.8
2	19.9	22.4	
3	20.3	22.2	24.2

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[1] Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. Med. Sci.*, 1935, **190**, 501.

bubbled through the plasma for 1 minute. No significant change took place in the prothrombin time under these conditions, in agreement with the findings of Andrus, Lord and Kauer.[2] Exposure of fresh dog plasma, centrifuged under oil, to CO_2 gas for 1 minute likewise does not produce a significant change in prothrombin activity. Dilution of the plasma with an equal portion of physiological salt solution increases its prothrombin time, which is not altered by subsequent exposure of this diluted plasma to CO_2 gas for 1 minute.

3. Effect of oxygen or CO_2 on the prothrombin activity of plasma previously exposed to an air current.

In carrying out the tests above described one must guard against blowing any of the reagents into the tubes with one's breath and thus inadvertently increase the CO_2 content of the plasma being tested.

Though the H ion concentration of the plasma, when outside of the limits of 7-8, definitely interferes with prothrombin activity, the restoration of activity by CO_2 to partially evaporated plasmas does not seem to depend entirely on a displacement of pH to the normal range.

Andrus, Lord and Kauer[2] found that in blood leaving the lungs there was about 10% less prothrombin than in blood entering them, and concluded that prothrombin was destroyed in the lungs. Loss of CO_2 in the aerated blood probably accounts for the difference observed

TABLE II.

August 4, 1941. Time of clotting of a mixture of 0.1 cc of plasma, 0.1 cc rabbit brain thromboplastin and 0.1 cc 0.025 M $CaCl_2$. O or CO_2 driven by pressure over surface of the plasma, in a closed vessel.

	Prothrombin time (sec)			
Type of plasma	Before evapora.	After evapora.	Oxygen for 1 min	CO_2 for 1 min
Rabbit, Citrated* (.3%)	9.3 (pH 7.9)	12.3 (pH 8.9)	12.5 (pH 8.9)	9.6 (pH 7.4)
Human, Citrated† (.3%) Dilut. 1-1 with phys. s.s.	17.4 (pH 7.6)	21.3 (pH 8.2)		17.3 (pH 8.0)
Hemophilic, Citrated‡ (.3%)	14.7 (pH 7.7)	75 (pH 9)	70 (pH 9)	15.3 (pH 7.4)
Dog, Oxalated§ (.13%)	9.7	74		

*Stream of moist, CO_2-free air run over the plasma for 1 hr (Loss in plasma volume by evaporation: 8%).

†Stream of dry air run over plasma in tube, 13 mm in diameter, for 2 hours. (Loss in volume by evaporation: 18%.)

‡Stream of dry air run over plasma in a wide bottom flask for 35 min. (Loss in volume by evaporation: 65%.)

§Exposed in a shallow vessel to a fan at 20 cm distance for 3 hours. (Loss in volume by evaporation: 65%.)

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[2] Andrus, W. DeW., Lord, J. W., and Kauer, J. T., *Science*, 1940, **91**, 48.

between the two samples. It may be anticipated, furthermore, that asphyxia or hyperventilation (as by forced breathing) will lead to significant fluctuations in the prothrombin activity of the blood. Loss of CO_2 may also account in part for the diminution in prothrombin activity of plasma after being heated to 56°C for 15 minutes, or after standing at 5°C for several days.

Summary. Exposure of citrated or oxalated plasma to an air current is followed by a rapid diminution in its prothrombin activity; this is corrected by the addition of CO_2 and not affected by oxygen.

13532

Hormone-Induced Ovulation in Domestic Fowl.

R. M. Fraps and G. M. Riley.

From the Bureau of Animal Industry, Department of Agriculture, Beltsville Research Center, Beltsville, Md.

Multiple ovulations in hens have been effected in the laboratories of the Bureau of Animal Industry by intravenous administration of a luteinizing preparation after pretreatment with pregnant mare's serum. So far as the authors are aware, this is the first demonstration of direct experimentally induced ovulation in birds.

Ovulation has been induced in a number of mammalian species by administration of anterior pituitary hormones or anterior pituitary-like hormones.[1] Kirschbaum, *et al.*,[2] and Witschi[3] have described full ovarian function in small caged passerine birds following prolonged stimulation with pregnant mare's serum. Increase in rate of ovulation is implied in the results of Clark,[4] Gutowska[5] and Dubowik,[6] but Bates, Lahr and Riddle[7] clearly demonstrated that doses of follicle-stimulating hormone or pregnant mare's serum sufficient to cause marked follicular growth in the laying hen and pigeon also caused cessation of ovulation, usually by the fourth day. These results of Bates, *et al.*,[7] have been confirmed repeatedly in this laboratory.[8] Leonard[9] suggested that luteinizing hormone, the presence of which he demonstrated in chicken pituitaries, possibly played a rôle in ovulation in birds.

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[1] Hartman, Carl G., Chapter IX, pp. 630-719, in *Sex and Internal Secretions*, Second Edition, Williams & Wilkins, Baltimore, 1939.

[2] Kirschbaum, A., Pfeiffer, C. A., Van Heuverswyn, J., and Gardiner, W. U., *Anat. Rec.*, 1939, **75**, 249.

[3] Witschi, Emil, *Wilson Bulletin*, 1935, **47**, 177.

[4] Clark, L. N., *J. Biol. Chem.*, 1915, **22**, 485.

[5] Gutowska, M. S., *Quart. J. Exp. Physiol.*, 1931, **21**, 197.

[6] Dubowik, I. A., *Arch. f. Exp. Path. u. Pharmakol.*, 1930, **158**, 154.

[7] Bates, R. W., Lahr, E. L., and Riddle, O., *Am. J. Physiol.*, 1935, **111**, 361.

Materials and Methods. White Leghorn hens in laying condition and housed in individual laying cages served as experimental subjects. Hourly laying records were maintained from 8:00 a.m. to 4:00 p.m. during the experimental period. Daily palpations through the cloaca were made to determine the presence or absence of eggs *in utero*.

Each hen was injected daily subcutaneously for 6 to 11 days with pregnant mare's serum* (PMS) in doses of 100 or 200 rat units according to labelled potency. If the daily doses of PMS are too low the desired interruption of ovulation does not occur, and if markedly stimulating doses are continued beyond 10 or 12 days the larger follicles undergo regression.[8] These observations provided the basis for the dosages and number of days pretreatment with PMS.

The luteinizing preparation (LH) was a fractionated extract from horse pituitaries.† The LH potency of the extract was determined by increase in weights of the seminal vesicles of male rats according to the method described by Fevold.[10] Full maturity of the immature mouse ovary following appropriate injection of the preparation indicated some contamination with follicle-stimulating hormone.

The intravenously administered dosages of LH effective in causing ovulation, 10 to 20 Fevold units, were determined in preliminary experiments. No attempt was made to determine the limits of LH effectiveness, and the dosages used are not necessarily optimal.

In the first experiments PMS injections were begun in 20 hens in one day, in 14 on the following day. LH injections were made subsequently at the same time into hens of both groups. Since no significant differences were observed in the reactions of hens receiving 6 or 7 injections of PMS before the first LH administration, treatment of and results in the two groups are presented together.

In the second experiment, 32 hens were given 20 units of LH intravenously after 8 or 10 days of PMS injections. Four similarly PMS-injected hens served as controls.

Ovulation was checked first by palpation through the cloaca to determine the presence or absence of an egg *in utero* and finally by postmortem inspection of the abdominal cavity for presence of yolks and of the ovary for presence of recently ruptured follicles. Diagnosis of ovulation following the first 2 LH injections in the first experiments depended wholly on palpation while all other diagnoses rested on use of all 3 technics.

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[8] Unpublished data.

[9] Leonard, S. L., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 566.

* "Gonadin Serum Veterinary" of Cutter Laboratories, Chicago.

† The extract was especially prepared by Chappel Laboratories, Rockford, Ill.

[10] Fevold, H. L., *Endocrinology*, 1939, **24**, 435.

Results. (1) *Repeated LH injections into PMS pretreated hens.* In these experiments each of 34 hens was injected daily with 200 units PMS. The first injection of LH was administered to 26 of these hens after 6 or 7 days PMS treatment, 13 hens receiving 10 units and 13 receiving 20 units. Ovulation occurred in 24 of the 26 LH-injected hens, as was shown by presence of single-yolked eggs *in utero* 22 hours later. Eight of the original 34 hens served as controls and continued to receive PMS but were not injected with LH; none of these hens had eggs in oviducts when the LH-injected birds were examined.

The second LH injections were made 22 hours after the first into the same 26 hens and at the same LH levels in individual hens. Seven of the LH-injected hens carried eggs *in utero* 24 hours later, while no eggs were found in oviducts of the 8 hens receiving PMS only. Each of the birds with eggs *in utero* following the second LH injection had ovulated following the first LH injection; the two hens failing to respond following either injection were killed at this time. One hen was found to have a highly stimulated ovary but with no follicles even approximately the size at which ovulation normally occurs. The other hen had ovulated twice, as was shown by the presence of two very recently ruptured follicles, but the yolk from neither ovulation had been picked up by the bird's oviduct.

The third LH injection was made into 17 of the 26 twice-injected hens 3 days after the second LH injection, all birds receiving 20 units of LH. Seven hens twice injected with LH served as controls against possible delayed ovulations consequent upon previous injections, and the original 8 hens receiving PMS daily were again carried without LH injection. Eight of the 17 LH-injected hens carried eggs *in utero* 24 hours later, while none of the hens in either group of controls had eggs in oviducts.

All birds were killed for direct examination of ovaries on the day following the third LH injection to the above 17 hens. The total count of recently ruptured follicles in ovaries of the 8 hens carrying eggs as a result of the third LH injection was 11. There was no evidence of recent ovulations in either group of controls.

The relatively small number of ovulating hens following second and third LH injections was probably due to ovulation of most of the reactive follicles in many hens following the first LH injections, a conclusion borne out by the results of single LH injections described below.

While these experiments, resulting in 42 positively identified ovulations, demonstrated that ovulations were effected following the injection of the luteinizing preparation into PMS pretreated hens, the exact or total effect of any single LH injection was obscured by the repeated treatments. Furthermore, the presence or absence of an egg in the oviduct could alone be used as a criterion of ovulation following the first and second LH injections, and the possibility of multiple ovulations following single injections could not be determined. The second group of experiments, in which PMS pretreated hens were killed the day following LH injection for direct examination of ovaries, was designed to clarify this possibility.

(2) *Ovulations following a single LH injection into PMS pretreated hens.* The conditions and the results of tests for multiple ovulations are given in Table I. As is shown in the table, all hens on the lower level of PMS, 100 units daily, were caused to ovulate by the LH injection with an average of 3.5 ovulations per hen. Thirteen of the 16 hens on the higher PMS level ovulated. Follicular regression had occurred in the ovaries of 3 non-ovulating hens, suggesting that the level of PMS pretreatment was too high.

The total number of proved ovulations following single injections of LH into the 32 PMS pretreated hens was 99 (Table I), or an average of 3.41 ovulations in the 29 ovulating hens. No ovulations occurred in the birds which received PMS alone.

Discussion. The data presented in this paper furnish a striking demonstration that ovulation of ovarian follicles in hens can be directly effected by injection of anterior pituitary preparations rich in the luteinizing hormone. Multiple ovulations thus induced are

TABLE I.
Ovulations Following Single Intravenous Injections of LH into PMS Pretreated Hens and Absence of Ovulations in Hens Receiving PMS Only.

	PMS pretreatment			Ovulating hens			Ovulations	
No. of hens	Daily dosage, rat units	Days	LH inj., units*	24 hr before LH No.	Following LH No.	%	Total	Per ovulating hen
16	100	8-10	20	0	16	100	56	3.50
16	200	10	20	0	13	81	43	3.31
4	100-200	11	0	0	0	—	0	—

*Determined by increase in weights of seminal vesicles of rats according to the method described by Fevold.[10]

in accord with observations of hormone-induced ovulation in mammals. By counts of ruptured follicles, 7 ovulations were effected in one hen, 6 in another, 5 in each of 6 hens, and 4 in each of 5. In a number of birds the full complement of ovulated yolks was recovered from the oviduct and body cavity. Recovered yolks ranged from 12.4 to 23.1 g in weight, a rather large range for these hens. Only a single egg was found in the oviduct of any hen following a given injection and only a single yolk was recovered from any egg.

The PMS pretreatment induced great follicular growth in most birds and mechanical factors may account partly for failure of the oviducts to include more yolks. However, it is unlikely that mechanical difficulties accounted entirely for the uniformly observed singly occurring one-yolked eggs in the oviducts of the LH-treated hens.

Summary. Ovulations were induced in hens by the intravenous injection of a luteinizing preparation into hens pretreated with pregnant mare's serum for 6 to 11 days. Ovulation was induced in some hens following each of 3 successive LH injections. As many as 7 multiple ovulations were effected in individual PMS pretreated hens following a single LH injection.

13533

Effect of Thyroidectomy, Castration, and Replacement Therapy on Thymus, Lymph Nodes, Spleen in Male Rats.*

WILLIAM O. REINHARDT AND PHILIP WAINMAN. (Introduced by Herbert M. Evans.)

From the Department of Anatomy and the Institute of Experimental Biology, University of California, Berkeley, Calif.

A quantitative relationship between the thymus gland and other lymphoid tissues has been shown to exist for the effects of castration[1] and adrenalectomy[2, 3] in the rat. Clinically, castration and hyperthyroidism are conditions in which the thymus and lymphoid tissues

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* Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation, New York City.

[1] Chiodi, H., *Rev. de la Soc. Arg. de Biol.*, 1938, **14**, 74.

[2] Reinhardt, W. O., and Holmes, R. O., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 267.

[3] Houssay, B. A., del Castillo, E. B., and Pinto, A., *Rev. de la Soc. Arg. de Biol.*, 1941, **17**, 26.

may vary from normal. The following experiments were undertaken to obtain a quantitative expression of these relationships. The effects of castration, thyroidectomy, castration and thyroidectomy, and treatment with thyroxin or testosterone propionate, singly or in combination with the operative procedures mentioned, have been studied in order to clarify any possible relationship in the reactions of the thymus, lymph nodes and spleen to the hormonal stimuli mentioned.

Methods. Male rats 60 days of age of the Long-Evans strain were divided into 4 main groups. Each group consisted of either unoperated normals, castrated, thyroidectomized, or castrated-thyroidectomized animals. Animals in each of these groups were in turn subjected either to treatment with thyroxin or testosterone propionate or served as normal or operated controls. A total of 12 groups (133 animals) constituted the experimental material.

Treatment was begun on the day following operation and continued daily for 35 days. The animals were autopsied at the age of 95 days, being sacrificed under chloroform anesthesia by bleeding from the heart.

Thyroxin was administered by subcutaneous injection of the crystalline product dissolved in alkaline solution in quantities of 7 or 50 μg per day (100 μg/cc). Testosterone propionate was administered subcutaneously in sesame oil (1.0 mg/cc) in amounts of 500 μg daily.

The animals received the regular stock diet XIV and were maintained under the same experimental conditions. Operations were checked for completeness at autopsy. Oxygen consumption tests performed on the thyroidectomized animals one week prior to autopsy gave further evidence of the completeness of operation.

At autopsy, thymus, spleen, cervical lymph nodes (superficial and deep) and mesenteric lymph nodes were dissected out in a routine manner by the same person to avoid variation, weighed, and fixed for histological examination, as in a previous study.[2]

Results. Table I presents the data obtained, those weights significantly different from the normal controls being italicized. Inspection of the table will show trends in weight of the thymus and lymphoid tissues of various groups, which, while not statistically significant according to the criteria employed, are of interest in their variation from the normal.

In general, it may be stated that castration alone or in combination with thyroxin treatment will cause definite increases in the weights of the thymus, lymph nodes and spleen. Testosterone pro-

TABLE I.
Weights of Thymus, Lymph Nodes and Spleen in Male Rats.

Type of animal	Treatment	No. of animals	Avg body wt, g	Thymus	Cervical nodes	Mesenteric nodes	Spleen
Unoperated	Controls	21	296	222 ± 13[*]	245 ± 19	358 ± 22	1135 ± 48
	Thyroxin (50)[‡]	7	285	235 ± 13	315 ± 21	390 ± 23	1122 ± 111
	Testosterone Propionate (500)	12	282	*121 ± 12*[†]	301 ± 17	*261 ± 12*	1070 ± 73
Thyroidectomized	Controls	14	263	183 ± 13	235 ± 16	386 ± 31	958 ± 74
	Thyroxin (7)	9	292	233 ± 22	250 ± 31	331 ± 16	1160 ± 76
	Testosterone Propionate (500)	7	245	*82 ± 9*	*167 ± 10*	325 ± 30	*630 ± 63*
Castrated	Controls	10	283	*419 ± 28*	*374 ± 34*	*449 ± 20*	*1384 ± 76*
	Thyroxin (50)	10	286	*510 ± 34*	*411 ± 29*	456 ± 30	1657 ± 199
	Testosterone Propionate (500)	10	284	*114 ± 14*	286 ± 25	365 ± 45	1187 ± 82
Castrated Thyroidectomized	Controls	10	194	218 ± 18	329 ± 25	323 ± 23	1080 ± 62
	Thyroxin (50)	12	231	301 ± 33	242 ± 50	402 ± 23	1210 ± 136
	Testosterone Propionate (500)	11	241	*84 ± 15*	197 ± 20	306 ± 29	1190 ± 153

[*] Mean wt in mg ± standard error of mean.

[†] In this study, those means showing a significant variation from normal controls are italicized. Significance was determined by calculation of a significant ratio greater than 3.0.

[‡] Figures in parentheses indicate the daily dose in micrograms.

pionate in combination with thyroidectomy caused decreases in weights of these tissues. Thyroidectomy prevented the marked increase in weight which followed castration alone. Thyroidectomy alone reduced the weights of thymus and spleen to a small extent. This reduction was repaired by the daily administration of thyroxin. Although not statistically significant, there is evidence of a positive additive effect of thyroxin treatment of castrated animals. The same may be stated of the weight decreasing effects of testosterone treatment of thyroidectomized animals. Testosterone propionate prevented the increase caused by castration, in the weights of the thymus, lymph nodes and spleen.

Histological examination was undertaken of representative hematoxylin and eosin stained celloidin sections of the thymus, spleen and lymph nodes of the various groups. Changes in the histology of the thymus after the above treatments are well known. Examination of the spleen in those groups showing significant increases in weight gave evidence of enlargement of the lymphatic nodules with coincidental hyperplasia of the diffuse white pulp. Changes in the lymph nodes, both cervical and mesenteric, were quantitative in nature, no specific morphological changes being noted.

In order of responsiveness to the stimuli applied in these experiments the tissues may be graded in order of decreasing reactivity (weight changes) as: thymus, cervical lymph nodes, mesenteric lymph nodes and spleen.

To determine whether changes in the thymus were positively correlated with similar changes in the lymph nodes, a correlation ratio was obtained between the thymus and the cervical lymph node weights employing the above and comparable data. A positive coefficient of correlation of 0.591 ± 0.080 was obtained. This would indicate that when changes occur in the one tissue, similar changes in the same direction are apt to occur in the other.

Summary. 1. Evidence is presented that there is a relationship in the reactions of the thymus, spleen, and lymph nodes to the effects of castration, thyroidectomy, and treatment with thyroxin or testosterone propionate, or combinations of these in the male rat. 2. Castration and thyroxin cause comparable increases in the weights of thymus, lymph nodes and spleen. Thyroidectomy and treatment with testosterone propionate tend to decrease the weights of these tissues. Thyroidectomy and testosterone propionate prevent the increase in the weights of thymus, spleen and lymph nodes which follow castration. The order of decreasing sensitivity to the stimuli employed is as follows: thymus, cervical lymph nodes, mesenteric lymph nodes and spleen. 3. A positive coefficient of correlation of 0.591 ± 0.080 was calculated from the data derived from the above experiments for the thymus and the cervical lymph nodes. This is further evidence of a similarity in reaction of these tissues.

13534

Electrophoretic Patterns of Concentrated Cerebrospinal Fluid.*

ELVIN A. KABAT, HAROLD LANDOW AND DAN H. MOORE.
(Introduced by Michael Heidelberger.)

From the Departments of Neurology and Anatomy and the Electrophoresis Laboratory, College of Physicians and Surgeons, Columbia University, and the Neurological Institute, New York.

The protein content of cerebrospinal fluid is of significance in the diagnosis of neurological disorders such as brain and spinal cord tumors, neurosyphilis, peripheral neuritis, etc. Very little is known, however, about the origin or composition of spinal fluid proteins, or of the variations in composition which accompany disease states. The Tiselius electrophoresis apparatus[1] has proven a valuable tool in the study of proteins, and has disclosed the presence in serum of 3 globulin fractions of different mobility. Variations in the electrophoretic patterns of human sera have been described in diseases such as lymphopathia venereum, multiple myeloma, etc.[2, 3, 4] It seemed possible that this method might be used in the study of the spinal fluid protein.

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* Aided in part by a grant from the William J. Matheson Commission.

Large volumes of cerebrospinal fluid obtained at encephalography and ventriculography were concentrated to a volume of about 2 ml by dialysis under pressure against saline in the icebox at 5°C to avoid denaturation. Concentrations of protein of from 0.4 to 1.5% or more could thus be obtained. These solutions were then dialyzed against 0.02 M phosphate buffer containing 0.15 M NaCl and studied in the Tiselius electrophoresis apparatus with a micro cell. Photographs of the migrating boundaries were taken by the Longsworth[5] method. Concentrations of protein and electrophoretic mobilities were calculated from the descending boundaries. Typical results are shown in Table I. Multiplying the total protein content of the original spinal fluid by the percent composition of any fraction gave approximate values for the amount of each component in mg per 100 ml.

Cerebrospinal fluid proteins show a pattern similar to that of the plasma proteins. When the total protein of the cerebrospinal fluid is elevated, all of the plasma components may be found (Fig. 1 A. No. 3). In those with normal levels of total protein, alpha globulin and fibrinogen may not be detected (Fig. 1 B. No. 2). The ratio of albumin to globulin is frequently somewhat higher than that found in serum; when the total protein of the spinal fluid is increased, the greater part of this increase is generally due to albumin. Alterations in the pattern of the serum proteins are, however, reflected in the cerebrospinal fluid; thus, in the 2 cases of lymphopathia venereum (4 and 5) it will be seen that the percentage of gamma globulin is high both in the serum and in the spinal fluid. Similarly, the albumin-globulin ratio is lowered in both, although this reduction in ratio is less marked in the spinal fluid.

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[1] Tiselius, A., *Faraday Soc.*, 1937, **33**, 524.

[2] Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, **70**, 309.

[3] Luetscher, J. A., Jr., *J. Clin. Invest.*, 1940, **19**, 313; 1941, **20**, 99.

[4] Gutman, A. B., Moore, D. H., Gutman, E. B., McClellan, V., and Kabat, E. A., *J. Clin. Invest.*, 1941, **20**, 765.

[5] Longsworth, L. G., *J. Am. Chem. Soc.*, 1939, **61**, 529.

TABLE I.

Electrophoretic Mobilities and Concentrations of the Protein Components in Cerebrospinal Fluid.

				Mobilities × 10⁻⁵					% composition						Conc. in original fluid (mg per 100 ml)						
															Total protein found	Calc. individual components					Colloidal gold curve
Case No.	Diagnosis	Cc fluid used	pH	A	α	β	φ	γ	A	α	β	φ	γ	A/G		A	α	β	φ	γ	
1	Chromophobe Pituitary Adenoma	46	7.4	6.0		3.0		0.5	58.4		23.4		18.2	1.4	31	18		7		6	1100000000
2	Idiopathic grand mal	60.0	7.3	5.9		2.9		0.7	62.4	6.7	13.4		17.4	1.7	31	19	2	4		6	,,
3	Polyneuritis	16.5	7.4	5.8	3.9	2.8	1.9	0.7	65.9	8.5	11.4	3.8	10.4	2.2	198	130	17	23	3	21	,,
4 (Serum)	Lymphopathia Venereum		7.5	5.0	3.2	2.3		0.6	39.6	8.4	17.7		34.3	0.65							
4	Lymphopathia Venereum	16.0	7.4	5.9		3.1		1.0	56.3		12.0		31.8	1.3	90	51		11		29	1111222100
5 (Serum)	Lymphopathia Venereum		7.3	4.8	3.3	2.9 2.4	1.6*	0.5	34.0	3.6	12.3	11.7*	38.4	0.5							
5	Lymphopathia Venereum	25	7.3	5.5	4.1	2.7	1.9	0.4	42.4	9.4	21.9		26.5	0.7	27	12	3	6		7	1100000000
6 (Serum)	Neurosyphilis		7.3	5.4	3.6	2.9 2.5		0.8	60.7	12.3	16.1		11.0	1.6							
6	Neurosyphilis	34.0	7.3	5.4	4.7 3.9	2.5		0.2	49.6	5.0	7.2		38.2	1.0	55	27	3	4		21	4334421100
7	Neurosyphilis	10.0	7.3	5.8				0.8	43.8				56.2	0.8	92	40				52	5555544210

*Not fibrinogen, but an unknown component.

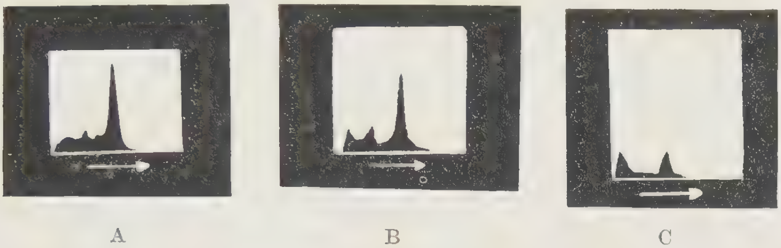

FIG. 1.

Electrophoretic patterns for cerebrospinal fluid (A) with high total protein content (case 3), (B) with normal total protein content (case 2), and (C) with abnormal colloidal gold curve (case 7).

Two cases of neurosyphilis whose spinal fluids showed abnormal colloidal gold curves, had a marked increase in the gamma globulin fraction. When the gamma globulin of case 7 (Fig. 1 C) was separated from the albumin electrophoretically, all of the colloidal gold reactivity was found in the gamma fraction and none in the albumin fraction. Although the colloidal gold reacting material is found in the gamma fraction, it may differ from the normal gamma component of spinal fluid since other fluids which contain equally large amounts of gamma globulin yield normal colloidal gold curves.

Summary. The electrophoretic pattern of the cerebrospinal fluid proteins resembles that of the plasma. Alterations in the composition of the serum proteins produce similar changes in the spinal fluid patterns. Colloidal gold activity in pathological fluids is associated with the gamma globulin.

13535

Inactivation of Sulfonamide Inhibitor by Azochloramid.

FRANZ C. SCHMELKES AND ORVILLE WYSS. (Introduced by N. Kopeloff.)

From the Research Laboratories of Wallace and Tiernan Products, Belleville, N.J.

The bactericidal effect *in vitro* of sulfonamide derivatives has been extensively investigated. Recently Neter[1] has reported that of a variety of bactericidal compounds which he studied only Azochloramid when used jointly with sulfonamides showed more than a mere additive effect upon the test organism.

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[1] Neter, E., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 303.

TABLE I.
Effect of Sulfanilamide and Azochloramid in a Synthetic Medium.
Inoculum 2,000,000 *E. coli*/ml.

Tube	Concentrations mg %		24-hr plate count × 000
	Azochloramid	Sulfanilamide	
1	0	0	182,000
2	0	5	185,000
3	0	15	163,000
4	.05	0	120,000
5	.10	0	93,000
6	.05	5	2
7	.05	15	0
8	.10	5	0
9	.10	15	0

We have examined this effect by quantitative methods. By plating out the contents of our mixed chemical-organism suspension we have obtained the following data: Two million *E. coli* per ml were inoculated into a synthetic medium and incubated for an hour at 37°C. The inoculated medium was then distributed in test tubes to which the drugs had been added in appropriate concentrations. The final drug concentrations and 24-hour counts are recorded in Table I.

A similar experiment with *Staph. aureus* with the exception that 20% sterile normal horse serum was added to increase the difficulty of killing, gave the results shown in Table II.

Similar effects upon the respiration of a growing culture were observed when using a Warburg respirometer as shown in Fig. 1.

The molecular ratio of the agents employed rules out any possibility that this synergism might be due to a chemical reaction between them, resulting in the formation of a more toxic compound. Neter's observations with other mixtures, particularly those obtained when examining the action of optochin and sulfapyridine on pneumococci, which two substances fail to show any signs of potentiation, make it extremely unlikely that the synergistic effect between Azochloramid

TABLE II.
Effect of Azochloramid and Sulfanilamide on *Staphylococcus aureus* in a Synthetic Medium + 20% Sterile Normal Horse Serum.

Inoculum of *Staph. aureus*, 2,000,000/ml

Concentrations mg %		Plate counts	
Azochloramid	Sulfanilamide	24 hr × 000	48 hr × 000
.4		33,000	126,000
	20	93,000	34,000
.4	20	10	120
Inoculated control		120,000	140,000

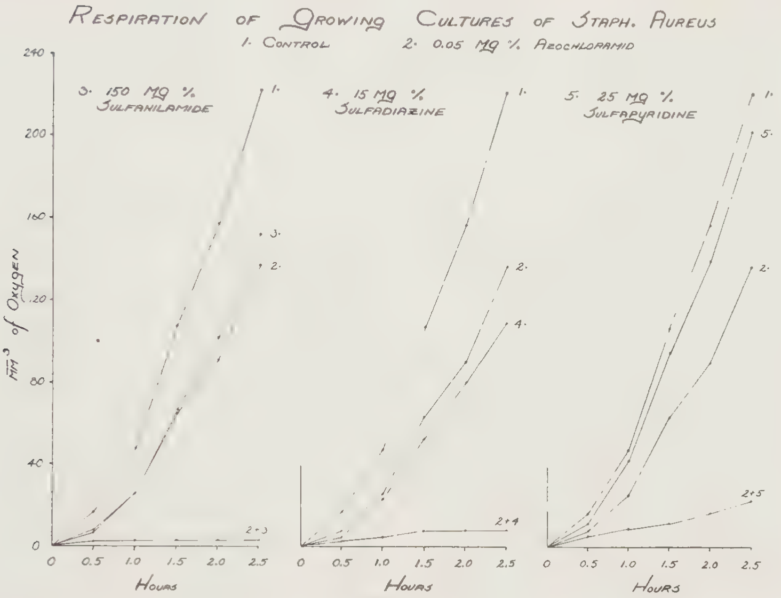

FIG. 1.

and sulfonamides could be due to a general lowering of the threshold for sulfonamide of the bacteria due to injury by Azochloramid.

TABLE III.
Effect of Azochloramid and Para-amino Benzoic Acid on the Inhibition of *E. coli* by Sulfanilamide.

Concentrations mg %			Turbidity (16 hr)
Sulfanilamide	Para-amino benzoic acid	Azochloramid	
0	0	0	34.0
0	0	.1	31.0
50	0	0	1.8
50	.1	0	33.5
50	.05	0	26.0
50	.01	0	2.0
50	.1	.1	1.5

TABLE IV.
Effect of Azochloramid and Peptone on the Inhibition of *E. coli* by Sulfanilamide.

Concentrations			Turbidity (16 hr)
Sulfanilamide mg %	2% Peptone ml	Azochloramid mg %	
0	0	0	43.
50	0	0	2.5
50	.5	0	30.0
50	.1	0	5.0
0	.5	.1	42.0
50	.5	.1	2.6

The specificity of the synergistic effect as well as the extremely small concentrations of Azochloramid required, directed our attention to the possibility that the action of Azochloramid might be due to the inactivation of the sulfonamide inhibitor. That this might actually be the case is shown in Tables III and IV.

Other chlorine compounds give similar results. Here, however, depending upon the higher reactivity of the halogen compound employed, higher concentrations may be required.

Of particular interest is the action of sulfanilamide and Azochloramid upon *Staph. aureus*, an organism notoriously resistant to sulfanilamide. In Table II it is shown that in the presence of a minute amount of Azochloramid, which is practically ineffective of itself, sulfanilamide has a striking bactericidal effect upon a large inoculum of this organism. Likewise in Fig. 1-A a minute amount of Azochloramid permits an otherwise ineffective sulfanilamide concentration to inhibit respiration of a large inoculum of growing staphylococci. McLeod[2] has published several exploratory experiments, one of which shows that his strain of Staphylococcus was characterized by its ability to release into the medium relatively large amounts of inhibitor. This might account for the resistance of this organism to sulfanilamide. The induced ability of sulfanilamide to kill large inocula of this organism in the presence of Azochloramid may be due to the latter compound's ability to inactivate the native inhibitor of the Staphylococcus, enabling sulfanilamide to kill this organism.

McLeod also showed that in some "sulfonamide-fast" strains the resistance of the organisms is accompanied by a great increase in the production of inhibitor. An examination was therefore made of the effect that small concentrations of Azochloramid might have upon the sulfonamide resistance of a "sulfonamide-fast" strain. Such a strain was quickly obtained in the following manner: Inocula of *E. coli* decreasing gradually from 1 ml to 1 loopful were transferred to tubes of a synthetic medium containing 50 mg % of sulfanilamide. Only the largest inoculum yielded growth in the first transfer. With this culture the process was repeated, slowly increasing the sulfanilamide concentration in the medium up to 100 mg %. However, upon continued culture from the smallest inoculum which gave growth in the sulfanilamide medium a culture was obtained in 6 transfers that grew well from a small number of organisms in the presence of 100 mg % of sulfanilamide.

By the addition of .04 mg % Azochloramid the resistance of this

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[2] McLeod, C. M., *J. Exp. Med.*, 1940, **72**, 307.

TABLE V.
Effect of Azochloramid and Sulfanilamide upon Sulfanilamide-fast *E. coli.*
Inoculum 2,000,000 *E. coli*/ml.

Sulfanilamide mg %	Azochloramid mg %	Turbidity 15 hr
0	0	49
100	0	45
25	0	47.5
0	.04	44.0
5	.04	0.5

culture was destroyed and it was again rendered sensitive to 5 mg % sulfanilamide as is shown in Table V.

Comment. In order to increase the accuracy of these experiments low concentrations of these agents were studied. The time of exposure is thereby lengthened and inaccuracies due to the time factor are eliminated. In order to produce these effects with short contact periods as would be desirable for clinical application appropriately increased concentrations of the agents are required.

Summary. These data indicate that Azochloramid potentiates the bactericidal effect of sulfonamides. This effect may be due to the inactivation of sulfonamide inhibitors by the chlorine compound.

13536

Measurement of Mean Blood Flow in Arteries and Veins by Means of the Rotameter.*

DONALD E. GREGG, R. E. SHIPLEY, R. W. ECKSTEIN, A. ROTTA AND J. T. WEARN.†

From the Department of Medicine, Western Reserve University, Cleveland, O.

In experiments designed to determine the accuracy of the thermostromuhr,[1, 2] the need arose for a simple and accurate method by means of which blood flow in arteries and veins of anesthetized dogs could be immediately quantitated from moment to moment for a considerable time period. For this purpose the rotameter[3] seemed to be the instrument of choice. Since this device apparently has not been used in physiological studies, attempts have been made to adapt it to the measurement not only of mean blood flows in arteries and veins but also of cardiac input.

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* The expenses of this investigation were defrayed by a grant from the Commonwealth Fund.

† We wish to acknowledge the help of Drs. A. S. Weisberger, E. F. Schroeder, and Mr. D. Book in some of these experiments.

[1] Gregg, D. E., Pritchard, W. H., Eckstein, R. W., Shipley, R. E., Steege, T. W., and Wearn, J. T., *Am. J. Physiol.*, in press.

[2] Shipley, R. E., Gregg, D. E., and Wearn, J. T., *Am. J. Physiol.*, in press.

[3] Schoenborn, E. M., and Colburn, A. P., *Transactions of the American Institute of Chemical Engineers*, 1939, **35**, 359.

Apparatus. The rotameter (Fig. 1, A and B) consists of a vertical transparent graduated tube (H) of constant taper within which is a small freely movable "float" (C or DEF). The height of the float in the tube is determined by the balance of the downward force (weight of float minus weight of displaced fluid) and the upward force (pressure drop across float times the maximal cross-sectional area of float). Increase in rate of flow disrupts this balance by increasing the pressure drop across the float, causing it to rise until the increase in the annular orifice reduces the pressure drop across the float to that existing at the previous flow level.

To insure minimal viscous drag all tubes and their connections should be of maximal diameter and minimal length, while the floats should be of minimal diameter and specific gravity. The rotameter design will be determined in part by the pressure and flow ranges in the vascular system in which flow is to be measured.

Critique of Apparatus for Arterial Flows. The float used for most flow ranges is usually made of stainless steel. Because of the viscous drag of blood on the float and tube a measurable part of the pressure head is permanently lost. The amount varies with each

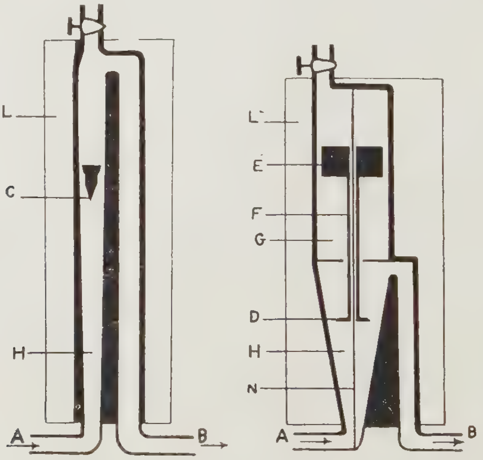

FIG. 1-A FIG. 1-B

Diagram of the rotameters used for measurement of mean blood flow in arteries and veins. *Part A,* rotameter used to measure small flows. *Part B,* rotameter with minimal viscous drag used to measure larger flows. *L,* Lucite jacket. Description in text.

rotameter ensemble and can be reduced by the use of lighter (aluminum) floats. Tests made on a rotameter (designed to measure coronary or femoral artery flow) indicate a pressure loss not exceeding 60 mm of water (Fig. 2, Curves 1 and 2). Such rotameters, as studied in a pump system reduce flow through themselves by not more than 3-4%. For larger flows through larger rotameters the flow reduction is even less.

In a pump system the mean float reading is not significantly affected by alterations in "heart rate" or stroke volume. Likewise, variations in direction and magnitude of the phasic rate of flow (recorded by the orifice meter[4]) even exceeding physiological limits are ineffective (Fig. 3, Part A).

However, it should be recognized that in any set-up the height of the float can be influenced by factors other than the rate of flow. Changes in the specific gravity of the blood introduce detectable but

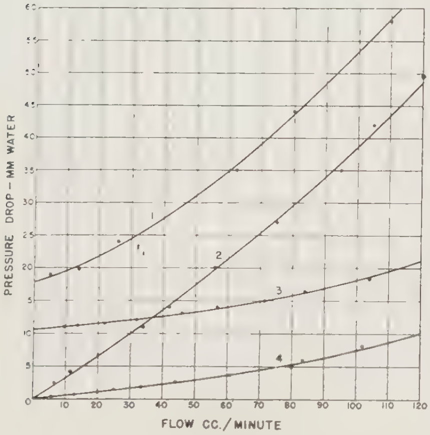

FIG. 2

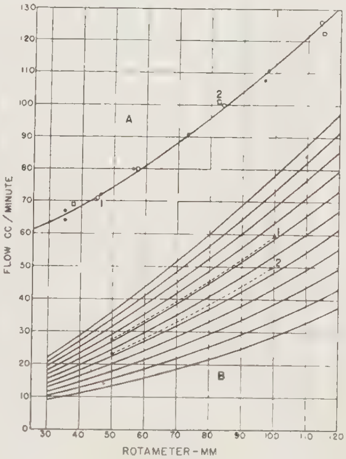

FIG 3

FIG. 2.

Curves 1 and 2. Data obtained in gravity system with blood showing pressure loss at different rates of flow in small rotameter with (Curve 1), and without (Curve 2) stainless steel float. *Curves 3 and 4.* Similar to Curves 1 and 2 but obtained in larger rotameter with (Curve 3) and without (Curve 4) aluminum float. (For Curves 3 and 4, flow values × 10.)

FIG. 3.

Part A. Graph showing fidelity of rotameter in recording mean flow in the presence of flow pattern variations. Flow patterns are: linear, those which resemble a carotid, femoral, or left coronary artery pattern and those with very large periods of backflow and zero flow. Dots, control points with all forward flow. Circles, flow patterns containing backflow up to 30% in volume. Squares, flow patterns containing periods of zero flow up to 20% in time. Point 1, 30% backflow. Point 2, 20% zero flow. *Part B.* Curves 1 and 2, rotameter calibrations of same blood at different temperatures; Curve 1 at 35°C, Curve 2 at 28°C. Solid curves represent series of *in vitro* calibrations of a rotameter (Fig. 1-A) obtained in a gravity system with bloods of different viscosities. Description in text.

not significant errors in reading (less than 1%). Alterations in specific viscosity of blood over the physiological range of 3.7-7.0[5] and induced by change in temperature or composition of blood (hemoconcentration or dilution, or the addition of certain anticoagulants) will give correspondingly different calibration curves for a rotameter (Fig. 3, Part B). For larger flows in larger vessels the viscosity effect may be almost entirely eliminated by use of a float similar to that described by Fischer, *et al.*,[6] the major part of which is removed from the flowing stream with high velocity to a stagnant region of no velocity. This (Fig. 1-B) consists of a brass disc about ⅛ mm thick and a brass cylinder connected by a small thin-walled glass tube of such length that the disc (D) is in the flowing stream and the cylinder (E) in the stagnant area (G), the float assembly being guided by a fine wire (N).

Critique of Apparatus for Venous Flows. For flows in the large and small veins or for measuring cardiac input (flow through both venae cavae) floats similar to those for arterial flows may be used, but those made of aluminum are more satisfactory. Tests made with blood in a gravity system indicate a pressure loss varying from 11-18 mm water as the flow changes from 1 to 1000 cc/min (Fig. 2, Curves 3,4). When flow through the jugular vein or both venae cavae (in open chest dogs) is measured by this apparatus the venous pressure may rise by 5-15 mm water. Such apparently offers no serious impediment to the venous circulation.

Calibration. Since the height of the float in Fig. 1-B is determined almost entirely by changes in rate of flow alone, one set of *in vitro* calibration points with blood will establish the calibration curve accurate to ±5 to 10% and applicable to float readings taken during any experiment.

Since, in a given animal experiment, the height of the float in Fig. 1-A is dependent upon the blood viscosity as well as rate of flow, the calibration curve of the rotameter which is applicable to that experiment may be determined by one of the following procedures with an error of less than 10%: (1) A series of float and flow readings taken during an animal experiment will establish the calibration curve for that experiment. (2) At the close of an experiment the rotameter is calibrated with the same dog's blood adjusted to body temperature. Such a calibration will correctly interpret float readings taken during the experiment provided the nature

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[4] Gregg, Donald E., and Green, H. D., *Am. J. Physiol.*, 1940, **130**, 114.

[5] Eckstein, R. W., Book, D., and Gregg, Donald E., *Am. J. Physiol.*, in press.

[6] Fischer, K., Blechman, S., and Lipstein, E., *Transactions of the American Institute of Chemical Engineers*, 1940, **36**, 857.

of the experiment does not involve significant viscosity changes. (3) During an experiment, blood leaving the rotameter may be temporarily diverted into a graduate and one flow and float reading taken. By observing the position of this one point, a calibration curve (or interpolation) may be immediately chosen from a previously determined *in vitro* series covering the range of specific viscosities found in different dogs (such as the series shown in Fig. 3-B) which will apply to float readings taken during the course of the experiment. Any calibration curve (as shown in Fig. 3-B) which has been obtained with different blood but of the same viscosity as that known to exist during an experiment may not always be used to determine the flow in that experiment, since in many tests it has been found that bloods of the same specific viscosity (as determined by a capillary viscosimeter) may not have the same calibration curve.

In animal experiments a combination of heparin [1000 units/kilo and pontamine fast pink BL (150 mg/kilo)] usually prevents the accumulation of fibrin on the rotameter float. In long experiments the anticoagulant injection should be repeated. Heparin alone, except in very large doses, is inadequate to prevent such deposition for a sufficiently long experimental period.

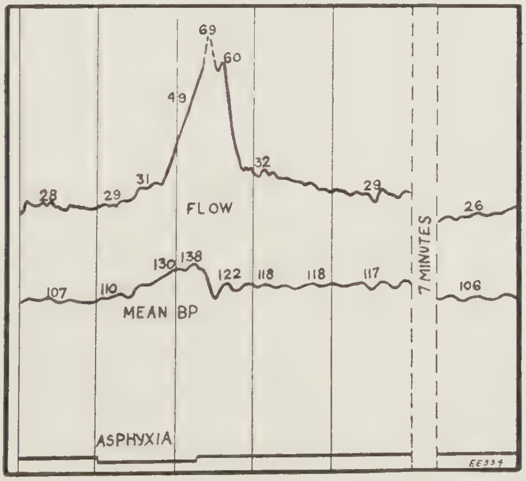

FIG. 4

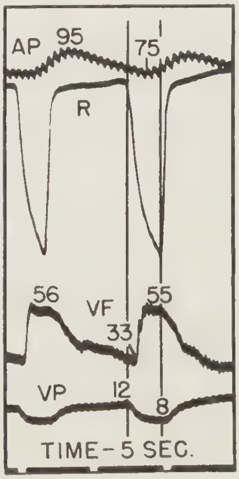

FIG. 5

Fig. 4.

Tracing of original kymograph record showing changes in mean blood flow (cc per minute) as recorded by rotameter in the left coronary artery and induced by temporary asphyxia. Vertical intercepts, one-minute intervals.

Fig. 5.

Reproduction of optical record showing effect of natural inspiratory attempt (trachea closed) on femoral vein flow as recorded by rotameter. AP, mean femoral artery pressure. R, respiration (vertical intercepts mark beginning and end of inspiration). VF, femoral vein flow in cc per minute. VP, mean femoral venous pressure.

Registration. (1) The height of the float indicating rate of flow may be visually noted from time to time. (2) Its position may be recorded on kymograph or photographic paper by means of a sighting attachment which is manually made to follow the movements of the top edge of the float.

Typical Records. Reproductions of original records are presented, showing the augmentation of blood flow in the left circumflex artery during temporary asphyxia (artificial respiration removed), Fig. 4, and in the femoral vein during an inspiratory attempt (trachea closed) Fig. 5.

Summary and Conclusions. The rotameter has been used to measure cardiac input and mean blood flow in the arteries and veins of the anesthetized dog. Typical records are shown. Tests indicate that in routine use the instrument will give reliable blood flow values with an error of less than 10%. Its use enables the experimenter to determine at a glance the moment to moment flow during the time that flow is actually being measured, an advantage not possessed by any other known method. The rotameter is so simple in operation that it should also serve a very useful purpose for the measurement of blood flows in student experiments in the classroom for which as yet no simple and reliable method has been available.

13537

Histamine Release in the Allergic Skin Reaction.*

GERHARD KATZ.

From the Department of Pharmacology, Tulane University of Louisiana School of Medicine.

It has recently been shown that blood cells from an allergic individual release part of their histamine into the plasma when they are exposed to the allergen *in vitro*.[1] This experiment on humans confirmed previous findings on blood from animals sensitized to egg-white.[2, 3, 4] Furthermore, it helped to substantiate the assumptions

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* Aided by a grant from the John and Mary R. Markle Foundation. Part of the experimental work was done in the Allergy Division of the Department of Preventive Medicine, Tulane University.

[1] Katz, G., and Cohen, S., *J. A. M. A.*, 1941, **117**, 1782.

[2] Katz, G., *Science*, 1940, **91**, 2357.

[3] Katz, G., *J. Pharm. and Exp. Therap.*, 1941, **72**, 22.

[4] Dragstedt, C., Ramirez de Arellano, M., and Lawton, A. H., *Science*, 1940, **91**, 617.

of the majority of earlier workers that histamine plays a rôle in the symptom complex of the allergic reaction.[5] While it was pointed out in the report referred to above that under certain conditions and to a certain degree such a release of histamine from circulating blood may contribute to the picture of the allergic attack, it may be assumed that histamine originating from the cells of the allergic "shock organs" (chiefly skin and mucous membranes) may take the major part in the humoral processes in allergy. The following is a report on a series of experiments in which an attempt was made to demonstrate that histamine is released in the allergic skin reaction.

In order to obtain tissue fluid for histamine determinations from parts of the skin exposed to allergen, an approach was employed which has been used by some authors for studies on cellular reactions in infectious diseases: production of the cantharides blister.[6] Application of cantharides to the human skin causes an exudation of tissue fluid between the stratum malpighii and the stratum corneum, which presents itself after a certain period of time as a well-defined blister with a minimum or absence of such inflammatory reactions as pain and hyperemia. In the experiments on allergic humans presented in this paper, the histamine content of fluid covering the stratum malpighii after application of cantharides was determined before and after introduction of the allergen.

Methods. The experiments were performed on volunteer patients with clinical ragweed allergy, none of whom had a history of serious allergic reactions, such as attacks of asthma. The dose of ragweed extract that produced a satisfactory skin reaction by intradermal injection was determined in each case. A cantharides plaster of from 2 to 4 cm in diameter was placed on the chest or upper arm and was removed after 15 to 24 hours. In the final method, which was adopted after some preliminary experimentation, the dead skin over the blister was removed. An inverted funnel, with the stem cut off, was then fastened over the denuded area by means of a holder which had previously been fastened to the intact skin with collodion. The funnel was filled with sterile saline, and, by means of 2 screws, pressure was applied from the holder to a degree which prevented fluid from leaking out and did not compress the skin more than necessary. The skin was warmed to 35°C (±0.5) by means of an infrared lamp. After flushing the funnel-covered tissue several times with saline by means of a syringe and needle, Locke's solution (0.8 to 1.0 cc) was introduced into the funnel and withdrawn and replaced with fresh fluid after constant contact periods.

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[5] Feldberg, W., *Ann. Rev. Physiol.*, 1941, **3**, 671.

[6] Kaufmann, F., *Klin. Wschnschr.*, 1928, **7**, 1309.

The samples thus obtained were either assayed directly on the isolated, atropinized guinea pig's ileum, or after extraction according to Barsoum-Gaddum and Code.[7] After a control period ragweed extract in saline was injected with a fine needle into the denuded area, in volumes of 0.03-0.05 and the saline samples were continued to be collected. A few control experiments showed that injection of this fluid volume *per se* does not lead to a detectable histamine release into the saline.

Results. Since the results were clearcut, and since the dose of the allergen producing these results usually caused some systemic reactions, only 14 experiments on 9 patients were done. The histamine content of the saline before application of ragweed was in some experiments below the threshold of the assay, and in the others slightly above it and decreasing in successive samples. The observation that tissues which are incubated with saline lose their extracellular histamine rapidly has been discussed in an earlier paper.[8]

In all experiments in which a strong reaction in the denuded area was produced by the ragweed extract, histamine in appreciable amounts was found in the saline. Within the first 10 minutes after introduction of the allergen, the histamine content of the fluid began to rise; it reached its maximum during the first 30 minutes, and it fell to or below the original level within 60 minutes. Roughly parallel with the increase in histamine in the fluid there was a considerable rise in protein as shown by trichloracetic acid precipitation. In three experiments it was determined quantitatively with the photoelectric colorimeter (Klett-Summerson). The results of a representative experiment are shown in Fig. 1, in which the amounts of histamine and protein released from one cm^2 of the denuded area before and after local injection of ragweed extract are recorded. The subject in this experiment suffers from ragweed hayfever for which he has not been treated. He had given a 3 plus skin reaction when tested intradermally with 1:10,000 ragweed extract a few days previous to the experiment. He received 10 times the above concentration and did not consider the ensuing systemic reactions particularly disagreeable. These consisted in an edema of the upper lids and throat irritation, which began 30 minutes after introduction of the allergen, and which were checked with epinephrine at the end of the experiment.

In 2 patients who had been receiving hyposensitizing treatment

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[7] Code, C., *J. Physiol.*, 1937, **89**, 257.

[8] Katz, G., *Am. J. Physiol.*, 1940, **129**, 735.

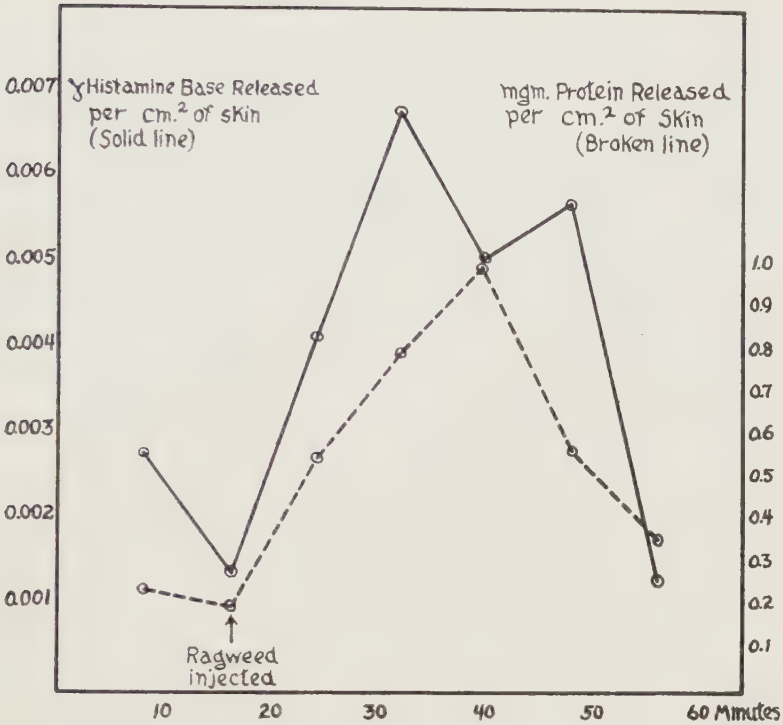

Fig. 1.

previous to the experiments, no local reaction was produced by injection of the allergen in a concentration equal to that with which they were treated at that time. Likewise, no histamine release was found. In 2 other, untreated, cases no histamine release was observed when the ragweed concentration was too small to cause a skin reaction.

Incidental Observations. In all instances in which a local skin reaction was produced, an increased fluid exudation from the denuded area was observed. Its maximum seemed to coincide with the time of the maximum histamine release. Its extent varied in different experiments. This aspect of the allergic skin reaction is being investigated with an improved technic. As in the previous experiments on histamine release in anaphylaxis,[3] the presence of a principle apparently with the properties of the "slow reacting substance" of Feldberg and Kellaway[9] was noticed. It caused a slow, delayed, contraction of the atropinized guinea pig's ileum, temporarily diminished the histamine sensitivity of this organ and was not completely eliminated by the chemical extraction process. However, since this substance was occasionally also found in small quantities in the fluid samples obtained before introducing the allergen, it is not clear whether or not it is a specific product of the allergic cell reaction. It might conceivably partly arise from the cell injury caused by cantharides.

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[9] Feldberg, W., and Kellaway, C. H., *J. Physiol.*, 1938, **94**, 187.

In a series of experiments an attempt to demonstrate a histamine release from the conjunctiva in positive ophthalmic tests was made. No increase in histamine was found in the tear fluid (obtained by blowing mentholated air against the eye) during the conjunctival hyperemia produced by ragweed. If we assume that this reaction is also histaminergic, the failure of these experiments may be due to the technical difficulties of obtaining tear fluid which has been long enough in contact with the tissue, or to a low permeability of the conjunctival membranes to histamine.

Discussion. These experiments show that in the local allergic reaction a substance with the properties of histamine is released from the skin, after the stratum corneum had been separated from the deeper layers through the action of cantharides. From these observations, it seems permissible to assume that this histamine release is likely to occur also in the allergic response of completely intact skin. The technic employed does not allow one to draw more than general conclusions concerning strength and duration of this histamine release, since the amounts of this substance appearing in the saline do not represent the amounts actually released in the deeper layers of the tissue. The rate of diffusion of histamine and perhaps also its inactivation in the skin interfere, possibly to a large extent, with getting an accurate picture of intensity and duration of the reaction in all parts of the skin involved.

The skin histamine release in concentrations which are pharmacologically active doubtlessly contributes, by producing the triple response, to the local change in skin function in the allergic reaction. However, as has been pointed out before, histamine is an important but not the only humoral agent resulting from antigen-antibody union.[3, 5] The liberation of other substances, such as acetylcholine, potassium, and the "slow reacting substance" seem worthy of investigation with the method described in this report.

The increased protein content of the saline during the allergic skin reaction probably had its origin in the well-known increased permeability of the capillaries. Here, as in other aspects of the allergic tissue response, it is not yet clear whether one is dealing with a primary action of the allergen upon the capillary walls, or with the action of histamine released from certain adjacent cells.

It seems probable that an interaction of both of these factors plays a rôle in most allergic tissue reactions.

The failure to obtain a release of histamine in assayable quantities in 2 patients previously treated with hyposensitizing ragweed injections tends to substantiate clinical observations that this treatment chiefly raises the threshold for the amount of allergen necessary to cause a histamine release, and not the threshold to released histamine.

Summary. Cantharides blisters were produced on the skin of allergic human subjects. After removing the stratum corneum over the blisters, the denuded areas were covered with saline, and the release of histamine and protein into the saline was determined before and after production of local allergic reactions by injection of antigen. Both the histamine and the protein content of the saline rose sharply within a few minutes after injection of the allergen and fell to the original level within 60 minutes. An increase in exudation of fluid and the appearance of a substance similar to the "slow reacting substance" were also observed.

13538

Acute Toxicity of Propionin in Rats and Mice.*

Harold C. Hodge.

From the Department of Biochemistry and Pharmacology, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.

Propionin is easily hydrolyzed by bacteria[1] and presumably also by the mammalian digestive tract; its toxicity may therefore be due to the effects of propionic acid or soluble propionates. Thus, Ozaki[2] reported that rats ingesting daily about 2.5 g of propionin per kg body weight died in 1 to 3 days. However, when rats were given about 1.4 g of propionin per kg per day, the only toxic effect was a marked regression of growth. Since these findings represent the current knowledge of the toxicity of propionin, this report is presented, not as an exhaustive study but to supply additional information on the acute toxicity of this compound.

Acute Toxicity for Mice. The detailed procedure will be given

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* This work was supported in part by a grant from the Carnegie Corporation of New York.

[1] Collins, M. A., and Hammer, B. W., *J. Bact.*, 1934, **27**, 473.

[2] Ozaki, J., *Biochem. Z.*, 1926, **177**, 156.

elsewhere. Propionin was administered intraperitoneally to groups of 3-months-old, male, albino mice (average weight, 20 g). From a statistical study of the data,[3] the dose to kill the average mouse is calculated as 1.7 ml of propionin (density of propionin, 1.1[4]) per kg body weight (Table 1-A). The mice remained quiet for a period of perhaps 10 minutes after receiving the intraperitoneal injection of propionin, then they became excited, squeaked and hopped around as if inebriated. Following the excitement, they became stuporous and sometimes died in less than an hour. It is interesting to note that propionin is somewhat more toxic than triacetin; the L.D. 50 of triacetin for albino mice (of comparable weight) was recently reported as 2.3 cc per kg body weight.[5]

Acute Toxicity for Rats. Propionin was administered by stomach tube to groups of albino rats of average body weight about 150 g. The dose required to kill the average rat is calculated as 15.3 ml of propionin per kg body weight (Table 1-B). After receiving propionin, the rats became quiet and appeared anesthetized for several hours. Those which recovered frequently showed a severe diarrhea. The data given are for deaths within 24 hours, a few of the rats which received doses of 2 ml or greater died on the second or third days.

TABLE I.

A. Mortality Following Various Doses of Propionin Given Intraperitoneally to Mice.

No. mice	Dosage, ml	No. dead	Mortality, %
24	0.03	8	33
24	0.04	16	67
24	0.05	22	92
24	0.07	24	100

For probit kill*—5.0, L.D. 50—0.034 ml.

B. Mortality Following Various Doses of Propionin by Stomach Tube to Rats.

No. rats	Dosage, ml	No. dead	Mortality, %
15	1.4	2	13
15	1.8	2	13
15	2.2	8	52
15	2.6	9	60
15	3.0	14	91

For probit kill*—5.0, L.D. 50—2.3 cc.

*"Probit kill—5.0" expresses in probability units, the figure corresponding to a 50% mortality. For details see 3.

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[3] Bliss, C. I., *Ann. Appl. Biol.*, 1935, **22**, 134.

[4] Simons, F. L., *J. Am. Chem. Soc.*, 1926, **48**, 1991.

[5] Li, R. C., Sah, P. P. T., and Anderson, H. H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 26.

Summary. Propionin injected intraperitoneally in mice has an L.D. 50 of 1.7 ml per kg. Propionin administered by stomach tube to rats has an L.D. 50 of 15.3 ml per kg.

13539

Non-Accumulation of Pectin Intravenously Injected into Rabbits.

EDWIN F. BRYANT, GRANT H. PALMER AND GLENN H. JOSEPH.
(Introduced by C. A. G. Wiersma.)

From the Research Department of the California Fruit Growers Exchange, Ontario, California.

A number of materials have been used as substitutes for blood in the treatment of shock and hemorrhage. Acacia solutions were used rather extensively for this purpose during World War I, mainly as a result of the work of Bayliss.[1] Later investigators have shown that there are many dangers attendant upon the intravenous use of acacia. Andersch and Gibson[2] reported that about one-half of the injected acacia accumulated in the liver. Yuile and Knutti[3] found that after repeated injections of acacia in dogs the liver weight increased 5 or 6 times and that sometimes as much as 8 to 10% of acacia (by weight) was found in the liver.

Pectin sols, having physical properties similar to those of acacia, should show the same advantages for transfusion purposes and yet, due to their different chemical constitution, might not cause damage by storage in the liver. The characteristic constituent of acacia, a gum exudate from certain tropical trees, is aldobionic acid which is unusually resistant to hydrolysis. Pectin, however, is a normal constituent of fruits and vegetables used for human food and is rather easily hydrolyzed in mildly acidic or alkaline systems and by many enzymes. Its molecule, with a weight of 150,000 to 300,000 is composed of chains of partially esterified galacturonic anhydride units.[4]

A general study of the effect of pectin in the circulatory system of rabbits has been in progress in this Department for the past 3 years. Various types of pectin in solutions of different concentrations have been injected intravenously into rabbits, particular attention being given to the effects upon organs involved with the circulatory mechanism. No ill effects of any kind have been observed. Chemical analyses have shown that there is no deposition of pectin in the liver and kidneys of rabbits receiving large amounts of pectin intravenously over a period of several weeks.

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[1] Bayliss, W. M., *Proc. Roy. Soc. (London) B.*, 1916, **89**, 380.

[2] Andersch, M., and Gibson, R. B., *J. Pharm. Exp. Therap.*, 1934, **52**, 390.

[3] Yuile, C. L., and Knutti, N. E., *J. Exp. Med.*, 1939, **70**, 605.

[4] Joseph, G. H., *Bull. Natl. Formulary Comm.*, 1940, **9**, 18.

Hartman, Schelling, Harkins, and Brush[5] of the Henry Ford Hospital in Detroit have recently proposed the use of pectin solutions as a blood substitute in the treatment of shock. Their preliminary experimental results as to the storage of pectin in the liver coincide with our findings. In view of the current interest in possible blood substitutes we submit some data from typical chemical analyses of liver, kidney and blood to supplement the work of Hartman and his associates.

Since the purpose, in our animal experiments, was to get excessive amounts of pectin into the blood, 6.0% pectin sols in normal saline were used. This is no doubt a greater concentration than would be desirable for transfusion purposes. Sols were made by dissolving 60 g of citrus pectin, which complied with the tentative standards of the forthcoming N.F. VII[6] in one liter of 0.85% sodium chloride solution. These sols were heated to 250° F for one hour, allowed to stand overnight to aid clarification, after which they were filtered into sterile containers. No attempt was made to adjust the pH of these sols to the region normal for blood. Although no discomfort has been noted as a result of administering pectin sols at their normal pH (3.2-3.8) some may wish to adjust the sols to higher pH values.

If pH adjustment of pectin sols is desired, it should be done after autoclave treatment because the molecular weight of pectin decreases rapidly at high temperatures when the pH is changed far from its natural region. Autoclaving the sols at their natural pH is usually just sufficient to bring the molecular weight down to the average molecular weight of human plasma proteins. Intravenous administration of unautoclaved pectin sols is probably not advisable because, due to the large size of the original pectin molecule, retention in body tissues might result.

The sols prepared as described above were injected in 10 ml amounts into the marginal ear vein, every other day, over the period from May 5 to July 7, 1941. The total volumes used were equivalent to approximately 15.0 g of dry pectin per rabbit. This amount of pectin for a 4 kg rabbit would be equivalent to about 25 liters of a 1.0% solution for a 70 kg human, an amount far in excess of any normal use.

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[5] Hartman, F. W., Schelling, V., Harkins, H. N., and Brush, B., *Ann. Surg.*, 1941, **114**, 212.

[6] Powers, J. L., and Beeler, E. C., *Bull. Natl. Formulary Comm.*, 1940, **9**, 31.

After completion of the 7 weeks' treatment the animals were autopsied. No abnormalities were found in the liver, spleen, kidneys, heart, lungs, or digestive tract. Analytical data on 4 rabbits so treated, together with 2 controls, are given in Table I. The method for determination of pectin in animal organs which we found reproducible and most satisfactory was developed by one of us (E.F.B.) from Youngburg's[7] method. The procedure is given below.

At autopsy the blood or organ is weighed and immediately frozen with dry ice. Before analysis, the organ is partially thawed, sliced paper thin with a sharp knife, and ground to a fine slurry in a mortar. Twenty-five grams of the slurry are extracted 3 times, each with 10 ml 20% trichloracetic acid for 5 minutes. The extracts are filtered into a 250 ml volumetric flask. The third acid extraction is followed by 2 hot water extractions. The residue on the filter is then washed with hot water until the flask is filled to the mark. After cooling to 20° C it is made to volume. Using 2.0 ml of extract (equivalent to 0.2 g of organ) and 5 ml of 85% phosphoric acid, the furfural distillation is made according to Youngburg. A small piece of paraffin in the distillation flask prevents foaming. Blanks on the organs of untreated animals should be deducted from the results. These blanks are similar to those found by Andersch and Gibson.[2]

The pectin used in our work formed 187.0 mg of furfural per gram, and since the method reveals as little as a few hundredths of a milligram of furfural, small amounts deposited in the organs could be detected.

As can be seen from the data in Table I, the furfural values for

TABLE I.
Furfural Recovery from Rabbit Organs After Repeated Pectin Sol Injections.

Rabbit		Total inj. (equivalent g dry pectin)	Mg furfural found per g of			
No.	Wt, kg		Liver	Kidney	Blood	Remarks
28	4.2	None	4.60	0.26	0.06	Control—No Pectin
29	3.4	None	3.68	*	*	” ” ”
20	4.2	15.0	4.30	0.26	*	Autopsied 3 days after last inj.
5	4.2	14.2	4.87	0.30	0.06	” 7 ” ” ” ”
27	4.2	14.1	6.38	*	*	” 3 ” ” ” ”
23	4.1	13.9	4.18	0.32	0.05	” 7 ” ” ” ”

*Not determined.

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[7] Youngburg, G. E., *J. Biol. Chem.*, 1927, **73**, 599.

the animals receiving pectin are not very different from those of the control animals. If pectin had been stored in the liver to the extent found by Andersch and Gibson[2] for acacia, the furfural values for the livers of the pectin-treated animals would have been of the order of 15 to 18 mg. No attempt was made to determine the time during which the pectin remained in the blood. An analysis was made 7 days after the last injection merely to show that the pectin had been eliminated from the blood.

Our work so far indicates that: (1) Massive amounts of pectin in the form of autoclaved isotonic solutions may be injected into the blood stream of rabbits over a period of some weeks without causing any noticeable effect upon the internal organs of the animals. (2) Chemical analyses showed no deposition of pectin in the liver and kidneys. (3) No pectin could be found in the blood 7 days after injection.

The authors are indebted to Mr. W. E. Baier and to Mr. T. C. Manchester of this Department for the aid they gave in certain phases of this work.

13540

Utilization of Parenterally Administered Casein Digest for Synthesis of Proteins.*

DWIGHT E. CLARK, ALEXANDER BRUNSCHWIG AND NANCY CORBIN.

From the Departments of Surgery and Medicine, The University of Chicago.

Intravenous or subcutaneous administration of casein digest (representing amino acids and polypeptides) as a means of parenteral nitrogen administration has been studied recently by Whipple and associates[1] in dogs previously depleted of plasma protein and on a low protein diet. Elman and Weiner[2] first reported that casein digests could be safely administered to man when certain precautions were observed. Clinical use of casein digests by parenteral administration in nephrotic children has been reported by Farr and associates,[3, 4] Shohl, Butler, Blackfan and MacLachlin,[5] and Shohl and

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* This work was facilitated by a grant from Mead Johnson and Company, Evansville, Indiana.

[1] Madden, S., Zeldis, L., Hengerer, A., Miller, L., and Whipple, G., *Science*, 1941, **93**, 330.

[2] Elman, R., and Weiner, D., *J. A. M. A.*, 1939, **112**, 796.

[3] Farr, L., *J. Ped.*, 1940, **16**, 679.

[4] Farr, L., and MacFadyen, D., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 444.

[5] Shohl, A., Butler, A., Blackfan, K., and MacLachlin, E., *J. Ped.*, 1939, **15**, 469.

Blackfan[6] have made extensive metabolic studies on infants receiving intravenous casein digest and crystalline amino acids.

Preliminary to clinical studies by the writers on the use of casein digests it was desired to ascertain whether the animal organism could actually regenerate plasma proteins with the intravenously administered digests as the *sole source of nitrogen* except for 15 to 30 mg of nitrogen injected intramuscularly in the form of liver extract (analysis of liver extract used showed a content of 15 mg of N per cc). Such a demonstration has not previously been reported as in other studies some protein was always present in the basic diets, although the amount might appear to be negligible.

The product used was an enzymatic hydrolysate of casein (Amigen) kindly furnished by Mead Johnson and Company of Evansville, Indiana, as a 10% solution. One hundred grams of the dried digest yield 12 g of nitrogen and it contains all the essential amino acids.[7]

Dogs were depleted of their plasma proteins by feeding the following nitrogen-free diet: 100 g dextrose, 50 cc olive oil, 50 cc "mineral milk," 10 gtts "Vi-Penta Oil" (Hoffman-LaRoche) containing Vitamins A, B_1, B_2, C, D.

The "mineral milk" was a formula modified after that published by McLance and Widdowson[8] and was made up as follows: Water, 5000 cc; KH_2PO_4, 204 g; calcium lactate, 337 g; $MgSO_4$-$7H_2O$, 65 g; $FeSO_47H_2O$, –? g; "iodized" NaCl (commercial), 200 g; copper sulphate, "pinch." The amount given daily supplied the mineral requirements adequately. Liver extract was injected in doses of 1 cc intramuscularly at one- to 2-week intervals throughout the entire periods of observation. The quantity of diet mentioned in the above paragraph supplied about 800 calories. The dogs were fed amounts equivalent to 80 calories per kg of body weight. During the depletion period they ate the diet readily. After the injection of casein digest began, which was carried out by syringe on an average of 5 to 7 times a day in 50 cc amounts, the appetite was frequently lost, necessitating forced hand feeding. This was carried out by placing a bolus of the aliment in the back part of the animal's mouth and inducing deglutition. While cumbersome, the method was satisfactory.

Plasma proteins were determined in duplicate by the micro-Kjeldahl-titration method.[9] The values for non-protein nitrogen were always within the normal range. Hematocrit determinations were made by centrifuging oxalated whole blood.

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[6] Blackfan, K., and Shohl, A., *J. Nutr.*, 1940, **20**, 305.

[7] Mueller, A., Kemmerer, K., Cox, W., Jr., and Barnes, S., *J. Biol. Chem.*, 1940, **134**, 573.

[8] McLance and Widdowson, *Lancet*, 1935, p. 1437.

In all, 7 dogs were employed. Each experiment consisted of a depletion period on the nitrogen-free diet and then an experimental period during which the basal nitrogen-free diet was supplemented with daily intravenous injections of the casein digest. In 2 dogs on which 2 experiments were performed an intervening period of stock feeding was given permitting protein regeneration in the

TABLE I.
Summary of Experiments on Plasma Protein Regeneration with Intravenous Injection of Casein Digest (Amigen).

Dog	Depletion period N-free diet, days	Duration of inj., days	Avg daily casein digest intrav. (10% sol.), cc		Beginning	End
					of inj. period	
Eddie	47	12	165	Wt, kg	6.7	6.1
Exp. I				Total PP*, g %	4.06	5.64
				Hematocrit	39	34.2
				RBC†, M	6.2	5.8
Eddie	21	17	177	Wt, kg	7.0	6.2
Exp. II				Total PP, g %	4.66	4.91
				Hematocrit,	41	32
				RBC, M	8	5.8
Fuzzy	27	21	248	Wt, kg	9.2	8.3
Exp. I				Total PP, g %	4.74	5.12
				Hematocrit	43	35
				RBC, M	7	5.0
Fuzzy	37	15	210	Wt, kg	9.1	9.2
Exp. II				Total PP, g %	4.31	4.97
				Hematocrit	43	35
				RBC, M	6.1	6.0
Pete	77	18	309	Wt, kg	7.4	6.6
				Total PP, g %	5.0	5.97
				Hematocrit	37	25
				RBC, M	7.5	4.7
Mike	21	11	256	Wt, kg	8.6	8.1
				Total PP, g %	3.98	4.99
				Hematocrit	38.2	27
				RBC, M	7.5	6.14
Kate	76	7	214	Wt, kg	8.1	7.6
				Total PP, g %	5.31	5.7
				Hematocrit	40	36
				RBC, M	5.5	6.1

*Plasma protein. †Red blood count.

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[9] Peters and VanSlyke, *Quantitative Clinical Chemistry,* Williams and Wilkins Company, Baltimore, 1932, **2**, 530.

normal physiological manner. The results of 7 experiments are summarized in Table I and show significant regeneration of blood plasma proteins.

In one dog not included in the table, which received the digest intravenously over a period of 33 days, the blood plasma proteins were 4.93 at the beginning of the injection period (Hematocrit 40.2, red blood count, 6.7 M) and 4.82 (Hematocrit 30, red blood count 5.2 M) at the termination with a loss in weight of .7 kg. A second dog was obviously ill during the entire injection period of 13 days during which time the plasma proteins changed from 4.69 (Hematocrit 32, red blood count 5.8 M) to 4.66 (Hematocrit 20, red blood count 4.3 M), the loss in weight being from 8.8 kg to 7.73 kg. Death of the animal terminated the experiment.

Conclusion. Regeneration of plasma proteins was found to occur in dogs with depleted proteins stores and lowered plasma proteins on a nitrogen-free diet receiving intravenously an enzymatic hydrolysate of casein as the sole source of nitrogen. Parenterally administered casein digest constitutes an effective method of parenteral nutrition.

13541

The Form of Combination of Radioactive Iron and Copper in Plasma Following Ingestion.

H. Yoshikawa,* P. F. Hahn and W. F. Bale. (Introduced by G. H. Whipple.)

From the Departments of Pathology and Radiology, University of Rochester School of Medicine and Dentistry.

It has been generally accepted as a fact that iron in transport in the blood plasma following its ingestion occurs in combination with globulin. The use of radioactive isotopes enables us to distinguish between iron† and copper‡ normally found and that recently added to the body economy. In earlier experiments we reported that, whereas about 80% of the radio iron in plasma shortly after feeding was in the filtrate following precipitation of the proteins by trichloracetic acid, nearly all of the iron in the red cell was in a form which was precipitated with this acid.[1] Below we tabulate further data relating to this subject.

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*Fellow of the Rockefeller Foundation.

† We are deeply indebted to Professor E. O. Lawrence and members of the Radiation Laboratory of the University of California for the radioactive iron used in these experiments, and in particular to Dr. M. D. Kamen, who was directly responsible for preparing the isotope.

‡ The radio copper was prepared in the cyclotron of the Department of Physics of the University of Rochester through the kindness of Dr. Gerhard Dessauer.

It can be seen that in a longer series of observations the fraction of iron in the plasma which is not precipitated by trichloracetic acid constitutes about 90% of the total iron (Table I) under the conditions of these experiments. Furthermore, the time after ingestion does not seem to be related to the amount of iron found in this fraction.

In Table II are listed the results obtained in an extension of these observations. By various procedures, the amount of iron in combination with protein was determined and found to be about 95% of the total. However, only about 15% of the iron was found to be actually bound to globulin as determined by half-saturation with am-

TABLE I.
Form of Combination of Radioactive Iron in Plasma Following Administration.
Trichloracetic acid precipitation of proteins.

Dog	Wt, kg	Hematocrit, %	Dose of iron isotope		Form of iron salt	Route	Time after administr., hr	Fraction of isotope in filtrate, %
			mg	counts/min.*				
37-227	4.7	27	84	45,800	$FeCl_3$	in diet	1	67
							2	77
							12	82
37-202	7.9	23	115	11,020	$FeCl_3$	” ”	1	100
							4	92
							6	98
							8	93
							10	94
37-202	11.9	19	58	25,400	Ferric ammon. citrate	gavage	0.5	98
							0.75	91
							1	96
							3	88
37-202	8.8	22	55	66,500	Ferric ammon. citrate		0.5	92
							1	91
							1.5	90
							2.5	85
38-112	10.0	26	122	12,400	Ferrous gluconate	intrav.	0.5	84
							2.0	92
	Average of all determinations							90

*Activity expressed as counts per minute on a scale of four Geiger Müller counter.

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[1] Hahn, P. F., Bale, W. F., Lawrence, E. O., and Whipple, G. H., *J. Exp. Med.*, 1939, **69**, 739.

monium sulphate. Since all but 10-15% of the iron appears in the filtrate following treatment with trichloracetic acid, it is likely that that iron which is not bound as globulin is in rather loose combination and is quite easily split off. We have not determined[§] whether or not this latter fraction of the iron is merely adsorbed or in combination as a salt. Only about 5% of the plasma iron was dialyzable against distilled water using cellophane tubes. When the plasma was acidified, on the other hand, about 60% of the iron dialyzed. When

TABLE II.
Form of Combination of Radioactive Iron in Plasma Following Oral Administration.

	Dog No.	40-149	39-196
	Date	6/17/41	7/7/41
	Dose, mg	0.93	4.1
	Activity, counts per min.	1980	7450
	Time after feeding, hr	1.7	2.2
	Red cell hematocrit, %	25.5	19.5
	Activity per 100 ml plasma	19.3	4.4
A	Protein-bound iron, precipitated by $(NH_4)_2SO_4$, % of total	93	98
B	Protein-bound iron, precipitated by 80% alcohol, % of total	74	94
C	Protein-bound iron, precipitated by 4 volumes of acetone, % of total	—	95
D	Globulin-bound iron, precipitated by ½ saturation with $(NH_4)_2SO_4$, % of total	17	15
E	Residue from dialysis against distilled water, % of total	—	8
F	Trichloracetic acid soluble fraction, % or total	79	—
G	Dialyzable iron, % of total	3.5	7
H	Dialyzable iron following acidification with sulphuric acid, % of total	59	—
I	Dialyzable iron following addition of 1 mg of ordinary iron as $FeSO_4$	—	17

Procedures used in treatment of plasma for determination of form in which iron is combined. References by letter are to Table II.

A. Protein-bound iron: 10 ml of plasma + 10 ml of water + 20 g of ammonium sulphate. Filtered.

B. Protein-bound iron: 10 ml plasma + 50 ml 95% ethyl alcohol. Allowed to stand for 30 min. Filtered.

C. Protein-bound iron: 10 ml plasma + 40 ml of acetone. Filtered.

D. Globulin-bound iron: 10 ml plasma + 10 ml of water + 20 ml saturated sodium sulphate. Filtered.

E. Residue from dialysis: See G below.

F. Trichloracetic soluble fraction: 10 ml plasma + 10 ml of 10% trichloracetic acid. Stood 30 minutes. Filtered.

G. Dialyzable iron: 10 ml of plasma dialysed against 200 ml of distilled water in ice-box for 24 hours.

H. Dialyzable acidified: 10 ml plasma + 10 ml of water + 10 ml of 0.3 *N* H_2SO_4 dialyzed in cellophane for 24 hours.

I. Dialyzable iron after addition of neutral iron: 10 ml of plasma + 1 mg of iron as $FeSO_4 \cdot 7H_2O$ allowed to stand overnight. Dialyzed against 200 ml of distilled water in ice-box for 24 hours.

[§] Dr. Yoshikawa's stay being interrupted, it was considered advisable to put on record the observations as they stand.

ordinary iron of the order of magnitude of 100 times the amount present in plasma was allowed to stand in contact with plasma containing some of the tagged iron, exchange took place to some extent since subsequent dialysis yielded 17% of the isotope.

While these studies were being carried out some preliminary investigations[2] concerning the metabolism of radioactive copper were under way and some effort to determine the form in which copper is transported in the plasma was indicated. These experiments are summarized in Table III.

Dog 40-133 used in these experiments was a normal animal, whereas dogs 40-149 and 40-179 were anemic, having been made so by

TABLE III.

Form of Combination of Radioactive Copper in Plasma Following Ingestion.*

		Dog	Time after feeding, hr	Activity conc. in plasma, total counts/100 ml	Activity conc. in fraction counts/100 ml	% of total
A	Protein-bound copper: Precipitated by four vol. of acetone	40-149	3.6	2700	2634	97
B	Copper precipitated by 5 vol. of 95% alcohol	40-133	2.4	3790	3045	80
C	Globulin-bound copper: Precipitated by half-saturation with $(NH_4)_2SO_4$	40-149	1.7	3700	975	26
		40-133	2.4	3790	570	15
D	Trichloracetic acid soluble copper	40-179	1.8	3460	2380	69
		40-133	4.2	3860	2500	65
E	Copper in diethyldithiocarbamate ether extr.	40-179	2.5	2800	1770	63
F	Dialyzable copper	40-149	1.7	3700	240	7

*Procedures used in treatment of plasma for determination of the form in which copper is combined. References by letter are to Table III.

A. Protein-bound copper: 5 ml of plasma + oxalate added to 20 ml of acetone and filtered.

B. Copper precipitated by alcohol: 2 ml of plasma + oxalate added to 10 ml 95% alcohol, allowed to stand 30 minutes and filtered.

C. Globulin-bound copper: 3 ml plasma + oxalate diluted with equal volume of saturated ammonium sulphate, allowed to stand 30 min. After centrifugation, the precipitate was washed with half-saturated ammonium sulphate before ashing.

D. Trichloracetic acid soluble copper: 3 ml of plasma + oxalate were diluted with 3 ml of water and 3 ml of 20% CCl_3COOH added. After 30 min. the mixture was centrifuged and the precipitate washed with 5% CCl_3COOH.

E. Diethyldithiocarbamate extracted copper: 5 ml of plasma + oxalate to which was added $Na_4P_2O_7$ solution. After addition of 1.5 ml of sodium diethyldithiocarbamate reagent the solution was extracted with ethyl ether repeatedly and the combined extracts ashed for counting.

F. Dialyzable copper: 3 ml of plasma + oxalate was dialyzed against distilled water at room temperature for 24 hours in a cellophane membrane.

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[2] Yoshikawa, H., Hahn, P. F., and Bale, W. F., *J. Exp. Med.*, in preparation.

repeated hemorrhage. The diets used consisted chiefly of white bread and salmon, providing a low iron intake, and have been described elsewhere.[2]

Nearly all of the copper is bound in some manner to protein. About two-thirds of the element is split off by trichloracetic acid and about the same amount is removed in the diethyldithiocarbamate procedure, so the binding is apparently not a very firm one. As in the instance of plasma iron, only a small amount of the copper is found tied to the globulin fraction of proteins. The fact that such a small amount of the copper is in the dialyzable fraction suggests that very little of the metal is likely in an ionizable form. Again, as in the case of the plasma iron, the data do not show whether we are dealing with true salt formation or whether the protein tie-up is one of adsorption.

13542 P

Inhibition in the Slow Muscle of the Scallop, *Pecten circularis aequisulcatus* Carpenter.

Andrew A. Benson, John T. Hays and Richard N. Lewis.
(Introduced by C. A. G. Wiersma.)

From the William G. Kerckhoff Marine Laboratory, Corona del Mar, and the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena.

The adductor muscle of Pecten consists of two parts, the larger of which is responsible for the rapid movements during swimming, and the smaller, which contracts slowly, is responsible for keeping the valves closed. The peculiarities of this muscle have been studied repeatedly.[1, 2, 3] A satisfactory answer has not been given to the question of how the slow muscle can withstand rather large tensions for indefinite periods without appreciable fatigue and at the same time be able to relax very quickly when necessary.

Reflex Relaxation. In Pecten stimulation of the mouth in a normal animal leads to rapid relaxation of the slow muscle. In our experience the sensitive area for this reflex is much wider than hitherto

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[1] Uexkuell, J. von, *Z. f. Biol.*, 1912, **58**, 305.

[2] Bayliss, L. E., Boyland, E., and Ritchie, A. D., *Proc. Roy. Soc.*, Series B, 1930, **106**, 363.

[3] Bozler, E., *Z. vergl. Physiol.*, 1930, **12**, 590.

reported, and includes all parts in the vicinity of, and innervated by the cerebral ganglion. Stimulation of the branchial nerves also leads to relaxation, although they do not connect directly with the cerebral ganglion, but with the visceral ganglion. That they nevertheless find their reflex center in the cerebral ganglion is shown by the fact that after cutting both cerebro-visceral commissures their stimulation elicits no relaxation of the slow muscle. As long as either commissure is intact, the reflex relaxation takes place. We were not able to find in this species the reported difference in action of the two commissures.[1, 2] As long as the nerve bands between the visceral ganglion and the slow muscle are intact, mechanical stimulation of these bands results in relaxation. This, too, is most likely reflex as this kind of stimulation becomes totally ineffective after cutting the bands.[2]

Peripheral Inhibition. The relaxation following a contraction produced by direct stimulation of isolated slow muscle is much slower than the relaxation observed when preganglionic mechanisms are stimulated. Pavlov[4] has claimed to have found peripheral inhibiting nerves in Anodonta which cause relaxation, but as Bayliss, *et al.*,[2] have pointed out, these experiments did not exclude the possibility of central inhibition. The muscles of the scallop are reported to be without an intrinsic ganglion system.[2, 5] If it can be shown, therefore, that some of the nerve fibers leading to the muscle have inhibiting properties, the existence of peripheral inhibiting fibers like those of crustacea[6] has to be accepted.

The slow muscle is innervated by two bands of nerves which run on the surface of the fast muscle from the ganglia and by other nerve fibers from the same source which lie in the region between these bands. It was found that by cutting the bands and the intermediate nerve fibers in different ways, preparations could be obtained with noteworthy properties. The most interesting of these is obtained by cutting every connection but the inside of one band. In such a preparation relaxation is obtained by any weak stimulation of the visceral ganglion itself, or of the more central elements of the nervous system. Also, after cutting all the nerves to the slow muscle, it has been possible to stimulate the peripheral ends of the band and obtain relaxation without the action of the ganglion.

If tone is increased in the slow muscle by any of several methods

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[4] Pavlov, J., *Pflüger's Arch.*, 1885, **37**, 6.

[5] Drew, G. A., "The Habits, Anatomy and Embryology of the Giant Scallop," Univ. of Maine Studies, No. 6, 1906.

[6] Harreveld, A. van, and Wiersma, C. A. G., *J. Exp. Biol.*, 1937, **14**, 448.

(direct stimulation, by pressing the muscle together or by forcibly keeping the valves closed) it is found that this tone can be made to disappear by the same types of stimulation just described.

It has been possible to a certain extent to study the effect of simultaneous stimulation of the contraction and relaxation mechanisms of this muscle. It was found that small contractions can be completely suppressed. Stronger ones persist but are diminished in size and are slower in development. The relaxation of such contractions is faster than that of uninhibited ones, and after a purely inhibitory stimulation contractions are depressed for some time, showing that the inhibition mechanism outlasts the stimulus to a much greater extent than does the contraction mechanism. This may well be due to the presence in the muscle of a substance which disappears slowly and causes relaxation.

These results aid in clarifying the mechanism of contraction and relaxation of the slow muscle in Pecten. It now seems reasonable to conclude that some type of peripheral inhibitory nerve fibers exists.

13543 P

Effect of Anoxia on Blood Sugar in Adrenalectomized-Pancreatectomized Dogs.*

Irvine McQuarrie, M. R. Ziegler and L. J. Hay.

From the Departments of Pediatrics and Surgery, University of Minnesota, Minneapolis.

In a previous investigation by the authors and their associates,[1] it was found that the blood sugar of adrenalectomized dogs was progressively decreased, frequently to hypoglycemic levels, as a result of their being made to breathe an atmosphere with a low oxygen content (5% to 9% O_2). In contrast with this response, normal animals in the post-absorptive state develop a sustained hyperglycemia[2] (as the dogs used in our previous experiments did before adrenalectomy[3]), when exposed to such an atmosphere. Since this latter reaction in the normal animal has been shown to be due to sympatho-adrenal stimulation with release of adrenalin into the blood stream and resultant mobilization of glucose from the liver, abolition of the hyperglycemic response to asphyxia would be expected to follow adrenalectomy. However, a well-defined hypoglycemic response, instead of a merely negative one, after adrenalectomy suggested the possibility of stimulation of the vago-insulin mechanism or a direct effect of anoxia on the islet cells of the pancreas with release of insulin. The experiments reported here were undertaken to obtain data bearing more directly upon this aspect of the problem.

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* Aided by a grant from the Graduate Medical Research Fund of the University of Minnesota.

[1] McQuarrie, I., Ziegler, M. R., Stone, W. E., Wangensteen, O. H., and Dennis, C., (a) Proc. Soc. Exp. Biol. and Med., 1939, **42**, 513; (b) *Chinese Med. J.*, 1940, **58**, 26.

[2] Kelloway, C. H., *J. Physiol.*, 1919, **53**, 211.

Dogs were depancreatized and adrenalectomized in two stages. The pancreas and one adrenal were removed at the first operation. After the animal had recovered satisfactorily from this procedure and had become well adjusted to a suitable diabetic regimen, the remaining adrenal gland was extirpated. Adrenal cortical extract (Upjohn) was then given subcutaneously twice daily in doses varying between 10 cc and 5 cc to prevent adrenal insufficiency until the operative wounds had entirely healed. Thereafter from 2 to 4 cc of the extract per day sufficed to prevent the usual manifestations of adrenal insufficiency. The basic daily diet contained the following ingredients per kilogram of the animal's body weight: casein 3, sucrose 9, egg yolk 2, egg white 2, artificial bone ash 0.4 and NaCl 0.5 g. This was supplemented by 100 g of ground, raw beef pancreas (or 10 g of lecithin), 2.5 mg ascorbic acid, .5 mg nicotinic acid, 2 mg thiamine hydrochloride and 3 drops of percomorph liver oil daily.

Following removal of the second adrenal gland, the daily insulin requirement fell from between 8 and 15 units to 1 or 2 units. Food, insulin and cortin were all withheld for about 16 hours before each acute experiment was carried out. Throughout each period of observation the animal was kept loosely fastened to a board in a specially adapted clinical oxygen tent, whether breathing room air or a mixture of 5% O_2 and 95% N_2. The tent was constantly maintained at atmospheric pressure. Eight control blood sugar curves were obtained on two adrenalectomized-depancreatized dogs while they breathed ordinary room air. Blood sugar determinations were made at 30-minute intervals for 2.5 hours. An equal number of acute experiments to determine the effects of anoxia on blood sugar were carried out on the same operated animals at intervals of 6 to 8

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[3] McQuarrie, I., and Ziegler, M. R., PROC. SOC. EXP. BIOL. AND MED., 1939, **39**, 525.

TABLE I.
Effect of Anoxia on Blood Sugar in Adrenalectomized-Depancreatized Dogs.

	Blood sugar, mg per 100 cc				
		Time in tent			
Dog No.	Initial sample	1 hr	1½ hr	2 hr	2½ hr
		Breathing room air.			
1	300	305	300	—	310
	355	346	—	300	330
	290	290	318	—	340
	320	250	—	260	270
2	265	240	—	240	250
	240	255	270	—	265
	305	250	—	265	246
	310	300	—	310	320
		Breathing 5% O_2 plus 95% N_2.			
1	360	380	—	340	345
	300	340	—	380	370
	352	348	348	—	355
2	252	280	—	320	300
	300	275	305	—	310
	300	345	380	—	360
	340	329	—	365	355

days, alternating with the control studies. In addition, the blood sugar responses to anoxia during periods of insulin hypoglycemia were determined in the animals before and again after removal of both the pancreas and the adrenals.

The results of the investigation may be summarized as follows (see Table I): There was no consistent rise or fall in the blood sugar levels of the adrenalectomized-pancreatectomized dogs, when they were exposed for 2.5 hours to an atmosphere containing but 5% O_2. The small fluctuations recorded did not differ significantly from those obtained for the same animals in room air. A composite curve representing the mean blood sugar values, determined at half-hour intervals throughout each of the 8 experiments, traversed an approximately straight horizontal line in the experiments involving anoxia as well as in those carried out in room air. Whereas sudden exposure to an atmosphere composed of 5% O_2 and 95% N_2 during a period of insulin hypoglycemia consistently caused a sharp rise in the blood sugar of normal dogs, no such alteration in the hypoglycemic curve resulted from anoxia in the depancreatized-adrenalectomized animals.

These results appear to indicate that anoxia normally stimulates both the vago-insulin and the sympatho-adrenal systems but that the former effect is masked under ordinary conditions by the more pronounced response of the adrenals. The stimulating effect on the insulin-releasing mechanism becomes evident only after the latter effect has been abolished. A similar interpretation was given by Gellhorn and his collaborators[4] to results of their interesting study on the mutual relationship of the vago-insulin and sympatho-adrenal systems under conditions of central excitation induced by anoxia and by metrazol. These investigators confirmed the hypoglycemic response to anoxia in adrenalectomized rats and found in addition that denervation of the adrenals had an effect similar to that of adrenalectomy. Metrazol, like anoxia, produced a hyperglycemia in normal but a hypoglycemia in adrenalectomized rats. They then found that intraäbdominal section of the vagi of adrenalectomized rats abolished the hypoglycemic response to either anoxic anoxia or metrazol. The effect of pancreatectomy in the present experiments was practically identical with that of vagotomy in their study.

Conclusions. Since pancreatectomy has been shown to abolish the hypoglycemic response of the adrenalectomized animals to anoxic anoxia, it may be concluded with Gellhorn that anoxia normally stimulates the parasympathetic (insulin response) as well as the sympathetic (adrenal response) system.

13544

Distribution of Biotin and Avidin in Hen's Egg.

PAUL GYÖRGY AND CATHARINE S. ROSE.

From the Babies and Childrens Hospital, and the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland.

Identification, with biotin,[1] of the curative factor (vitamin H) for egg-white injury in rats, and the recognition that avidin,[2] a protein-like constituent of egg white, is the injury-producing principle[3] of raw egg white raised the question of the distribution of these substances in whole egg. The search for an answer is especially appropriate when it is recalled that in the investigations of Kögl and Tönnis[4] biotin was isolated from egg yolk, whereas, on the other hand, raw egg white is the only known source of avidin.

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[4] Feldman, J., Cortell, R., and Gellhorn, E., *Am. J. Physiol.*, 1940, **131**, 281.

[1] György, P., Melville, D. B., Burk, D., and du Vigneaud, V., *Science*, 1940, **91**, 243; du Vigneaud, V., Melville, D. B., György, P., and Rose, C. S., *Science*, 1940, **92**, 62.

[2] Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **136**, 801; 1941, **140**, 535.

[3] György, P., Rose, C. S., Eakin, R. E., Snell, E. E., and Williams, R. J., *Science*, 1941, **93**, 477.

In the main, the problem is two-fold: 1. Does the whole egg contain a surplus of avidin or of biotin? 2. How can free biotin in egg yolk and excess of avidin in egg white exist simultaneously in the intact egg?

Experimental Method. Free biotin was determined by the yeast growth method.[5] Biotin bound to avidin (AB) was freed by steaming and then determined microbiologically as biotin. The concentration of avidin in excess is expressed in terms of its biotin-binding capacity.[2] The values obtained for free biotin in egg yolk were controlled by experiments on rats suffering from egg-white injury which were fed a diet containing a large proportion of egg white.[6] In the experiments with egg yolk special care was taken to avoid admixture of egg white (avidin).

Experiments. Group 1. Quantitative determinations of biotin and avidin in whole egg in which egg white and egg yolk were thoroughly mixed invariably showed an excess of avidin, as illustrated by two examples:

	Avidin total Free (A) + bound (AB) μg/cc	Avidin in form of biotin complex (AB) μg/cc	Excess of free avidin (A) μg/cc
(*a*)	.50	.19	.31
(*b*)	.54	.30	.24

These results were confirmed indirectly by experiments in which egg white, egg yolk (of one egg) and mixtures of yolk and white in the proportion found in the whole egg were analyzed, as shown by the following values:

		Biotin (free *plus* bound)			Avidin (free *plus* bound)		
		Found		Calculated	Found		Calculated
	Vol., cc	μg/cc	μg/total vol.	μg/total vol.	μg/cc	μg/total vol.	μg/total vol.
White	27	.15	4.1	—	.70	19.0	—
Yolk	14	.53	7.4	—	.0	.0	—
Whole egg	41	.30	12.3	11.5	—	22.0	19.0

Group 2. In Group 1 the biotin values for egg yolk were obtained

by using a suspension of egg yolk in saline solution without or with steaming. The possible objection that this method may leave part of the biotin content of the egg yolk unaccounted for has been investigated in special experiments. Furthermore, the egg yolk was subjected to treatment of various kinds before the quantitative microbiological tests for biotin were carried out. It was hoped that the results obtained might shed light on the form in which biotin occurs in egg yolk. A few examples are summarized in Table I, as illustrative of the results obtained.

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[4] Kögl, F., and Tönnis, B., *Z. Physiol. Chem.*, 1936, **242**, 43.

[5] Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 175; *Univ. Texas Pub.* No. 4137, 1941, p. 18.

[6] György, P., *J. Biol. Chem.*, 1939, **131**, 733.

The tentative conclusion may be drawn that suspension of egg yolk in cold saline solution gives values for biotin which are, as a rule, either equal to or only moderately lower than the values found in aliquot portions of the same saline suspensions after acid hydrolysis or after steaming. Equally high values were obtained in samples which were "extracted" after steaming with distilled water in varying dilutions. In contrast, the filtrates from suspensions of egg yolk in cold distilled water contained only traces of the biotin found in "hot" distilled water or in saline extracts or in the hydrolysates. Filtering with kieselguhr, as well as precipitation with acetone, diminished the biotin content of the saline extracts. Although the differences obtained in the biotin values in cold and steamed samples of egg yolk "extracts" in distilled water are reminiscent of the behavior of avidin in egg white, the fact that cold "extract" in saline gives values almost as high as those obtained after steaming cannot be reconciled with the assumption of an avidin-like substance in egg yolk. The results summarized in Table I speak rather in favor of the conclusion that biotin is present in egg yolk in combination with a larger molecule, probably a globulin-like constituent of the egg yolk. This compound can be precipitated by cold distilled water, partly also by acetone; it is soluble to a large extent in cold saline and can be split, with simultaneous liberation of biotin, by steaming for a short time. Biotin is available for yeast cells even in the form of the high molecular protein compound.

Biotin liberated from liver by hydrolysis is freely dialyzable,[7] as is pure biotin itself. If the biotin of egg yolk should be found to be present in the form of a high molecular compound, it should be undialyzable. That it is undialyzable has been proved unequivocally in experiments summarized in Table II and was also indicated by the recent investigations of Williams and his collaborators.[8]

Since this biotin of egg yolk is available for yeast cells, the

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[7] Birch, T. W., and György, P., *J. Biol. Chem.*, 1939, **131**, 761.

[8] Thompson, R. C., Eakin, R. E., and Williams, R. J., *Science*, 1941, **94**, 590.

TABLE I.
Content of Biotin in 3 Samples of Egg Yolk Subjected to Various Treatments.

		Biotin (μg/cc)		
	Treatment	A*	B*	C*
Saline dilution (cold)	1 cc egg yolk to 100 cc with saline:			
	(a) passed through Whatman filter paper No. 42†	.34	.60‡ .65‡	.32
	(b) filtered with kieselguhr		.02‡ .06‡	
Saline dil. (steamed)	1 cc egg yolk to 100 cc with saline, steamed 15 min:			
	(a) passed through filter paper No. 42†	.41	.60‡ .66‡	.59
	(b) filtered with kieselguhr	—	—	.54
Distilled water dil. (cold)	1 cc egg yolk to 10 cc with distilled H_2O:			
	(a) passed through filter paper No. 42 until clear as possible	—	.009‡ .02‡	.03
	(b) filtered with kieselguhr	—	—	.015
	1 cc egg yolk to 100 cc with distilled H_2O, passed through filter paper No. 42: filtrate clear but test for protein with sulfosalicylic acid positive	.037	.026	—
	1 cc egg yolk to 1000 cc with distilled H_2O, passed through filter paper No. 42: test with sulfosalicylic acid positive	.032	.06	—
Distilled water dil. (steamed)	1 cc egg yolk to 10 cc with distilled H_2O, steamed 15 min:			
	(a) passed through filter paper No. 42 until clear as possible	—	—	.52
	(b) filtered with kieselguhr	—	—	.41
	1 cc egg yolk to 100 cc with distilled H_2O, steamed 15 min:			
	(a) passed through filter paper No. 42 until clear as possible	.36	—	—
	(b) passed through filter paper No. 42†	.41	—	—
	(c) filtered with kieselguhr	.38	—	.52
Hydrolyzed sample	1 cc egg yolk to 10 cc in $6N$ H_2SO_4, autoclaved 1 hr neutralized, filtered through paper No. 42†	— — —	.63‡ .60‡ .65‡	— — —
Acetone extract	1 cc egg yolk to 10 cc with saline, precipitated with 4 times vol. of acetone, diluted with water and fat removed by means of ether (free from peroxides), water layer evaporated to dryness and tested for biotin	—	.26	.21

*Each vertical column represents a different sample of egg yolk.
†Before filtering, dilutions were adjusted to pH 3.5.
‡Same egg yolk, tested on different days.

question arose whether it is equally available for rats in the cure of egg-white injury. It was found that rats were completely cured of egg-white injury when they were given daily injections of suspensions of egg yolk in saline which contained amounts of biotin as

TABLE II.
Dialyzability of Biotin in Different Samples of Egg Yolk.

		Biotin (μg/cc)			
	Treatment	B*	C*	D*	E*
Saline dilutions (cold)	1 cc egg yolk to 100 cc with saline, dialyzed 24 hrs in cold room, steamed 15 min, filtered†:				
	Control‡	.68	.62	.69	—
	Dialyzed sample	.75	.58	.55	—
	Biotin added to egg yolk (0.2 μg/cc to B, 0.4 μg/cc to D); 1 cc egg yolk to 100 cc with saline, dialyzed 24 hrs in cold room, steamed 15 min, filtered†:				
	Control‡	.88	—	1.1	—
	Dialyzed sample	.53	—	.66	—
Saline dil. (steamed)	1 cc egg yolk to 100 cc with saline, steamed 15 min, filtered†, then dialyzed 24 hrs in cold room:				
	Control‡	—	—	.69	—
	Dialyzed sample	—	—	.035	—
Distilled water dilution (cold)	1 cc egg yolk to 100 cc with distilled water, filtered,† dialyzed 24 hr in cold room, steamed 15 min				
	Control‡	—	—	—	.023
	Dialyzed sample	—	—	—	.015

*Each vertical column represents a different sample of egg yolk. For other data on samples B and C, see Table I.

†Before being passed through Whatman filter paper No. 42, these dilutions were adjusted to pH 3.5.

‡Control samples were kept at the same temperature and for the same length of time as the dialyzed samples before being assayed.

low as 0.2, 0.1, and even 0.05 μg, as determined by the yeast growth method. The smallest amount (0.05 μg) proved to be approximately the "rat day dose."[9] Thus biotin in egg yolk, although bound in a high molecular, undialyzable form, is, physiologically at least, as active as pure biotin.

Conclusions. 1. Whole egg contains an excess of avidin, as the amount of biotin in the whole yolk is unable to neutralize the amount of avidin in the white of the same egg. 2. Biotin in egg yolk is present in a high molecular, undialyzable form which is physiologically active in yeast growth tests or in tests made on rats with egg-white injury.

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[9] György, P., Rose, C. S., Hofmann, K., Melville, D. B., and du Vigneaud, V., *Science,* 1940, **92**, 609; du Vigneaud, V., Hofmann, K., Melville, D. B., and György, P., *J. Biol. Chem.,* 1941, **140**, 643.

13545

Comparison Between Gravimetric and Reduction Methods for Determination of Pregnandiol Glucuronidate.*

OSCAR HECHTER. (Introduced by H. J. Deuel, Jr.)

From the Endocrine Research Division, Cedars of Lebanon Hospital, and the Department of Biochemistry, University of Southern California, Los Angeles, Calif.

The evaluation of luteal activity in the human has been facilitated by recognition of pregnandiol glucuronidate (P.G.) as a metabolic derivative of progesterone.[1] Quantitative evaluation of luteal activity, however, is not obtainable by P.G. estimation in the absence of quantitative information regarding the efficiency of the extra-ovarian factors necessary for the excretion of P.G.[2] Further, the presence or absence of urinary P.G. may not serve as a qualitative test for luteal activity, since it is possible to obtain zero values for P.G. in the presence of circulating progesterone.[3] Recognizing these difficulties, it is still possible to use P.G. determinations for useful information. The presence of urinary P.G. may be taken as strong suggestive evidence for functional lutein tissue and the quantity of P.G. excreted represents a minimal estimate of the amount of metabolized progesterone.

The practical utility of P.G. determinations for evaluation of luteal function presupposes that the method of P.G. estimation is satisfactory. In dealing with the small amounts of P.G. excreted by non-pregnant women, Venning's modified gravimetric method[4] affords no absolute guarantee that the material obtained by that procedure is actually P.G. The need for a quantitative check of the purity of the P.G. isolated by the method of Venning[4] has led to the development of a simple method of P.G. determination, which depends on the estimation of reducing activity obtained after acid hydrolysis of P.G. The results found with this reduction method are compared to P.G. values obtained gravimetrically.

Methods and Results. A sample of sodium pregnandiol glucuronidate (Na P.G.) (Ayerst, McKenna and Harrison) recrystallized from ethyl alcohol (M.P. 271°C uncorrected) was used in the de-

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* Aided by grants to the Endocrine Research Fund from Miss Norma Shearer, Mr. Harry Warner and the Schering Corporation.

[1] Venning, E. H., and Browne, J. S. L., *Endocrinology*, 1937, **21**, 711.

[2] Hamblen, E. C., Ashley, C., and Baptist, M., *Endocrinology*, 1939, **24**, 1.

[3] Venning, E. H., and Browne, J. S. L., *Endocrinology*, 1940, **27**, 707.

[4] Venning, E. H., *J. Biol. Chem.*, 1938, **126**, 595.

velopment of the method. Preliminary experiments revealed that maximal reducing activity was obtained by hydrolysis of Na P.G. with 0.2 ml of 10 N H_2SO_4 per cc of P.G. solution for 60 minutes in a boiling water bath; and that the hydrolysis of Na P.G. by acid under these conditions proceeds as well in 50% acetone as in water.

The calibration curve was obtained in the following manner: Known amounts of recrystallized Na P.G. dissolved in 50% acetone were added to hydrolysis tubes graduated at the 5 ml volume mark. Acetone and water were added so that the final volume was 3 cc and the acetone concentration was 33%. 0.6 cc of 10 N H_2SO_4 was added to the P.G. solution and the tubes were placed in a boiling water bath for one hour. A long piece of glass tubing, inserted through a rubber stopper, was used as a reflux condensor to minimize the vaporization of acetone during the hydrolysis. After the hydrolysis period, the tubes are removed, cooled and then neutralized as follows: 1 drop of phenolphthalein is added to each tube, 15 N NaOH is added drop by drop till the solution becomes colored, and then 1 N H_2SO_4 is added until 1 drop brings the solution back to the colorless state. Upon completion of the neutralization, the contents are diluted with water to the 5 ml mark, mixed, and then filtered to remove a white precipitate which appears during the course of hydrolysis. 2.0 ml samples of this filtrate are treated for Cu reducing activity according to the method of Folin and Wu,[5] adapted to the Leitz photoelectric colorimeter. The final blue solution is diluted to the 12.5 ml mark when less than 4 mg of Na P.G. is analyzed, and to the 25 ml mark when 4-8 mg are present. When light absorption is plotted against concentration the curve is almost linear from 0.25 to 8 mg.

This method is sufficiently sensitive to allow detection of as little as 0.25 mg of Na P.G. and is accurate to ±0.1 mg. It is recognized that higher sensitivity might be obtained by utilization of a more

TABLE I.

Comparison of Gravimetric and Chemical Determination of Pregnandiol Glucuronidate in the Urine of Normal Women.

Case	Pregnandiol Glucuronidate in mg per 24 hours		
	Gravimetric	By reduction	% impurity
1.	4.0	3.8	5.0
2.	3.0	3.0	0
3.	3.8	3.7	2.6
4.	1.6	1.6	0
5.	5.3	5.1	3.5
6.	4.8	4.4	8.3
7.	2.3	2.1	8.7

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[5] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

sensitive reducing procedure and that the accuracy might conceivably be increased. However, the qualitative information regarding the progesterone activity obtained from P.G. does not appear to warrant further quantitative refinements of the reduction method at this time.

Table I illustrates the comparative values of P.G. in the urine of 7 non-pregnant women when obtained by gravimetric analysis of Venning[4] and by the reduction method described above on the isolated precipitates of P.G. In general, it will be seen that there is good agreement between the two methods. However, this situation is not true in all cases. Table II gives results on 2 patients on whom aberrant results were obtained.

With patient 8, no luteal activity (as evidenced by the vaginal smear method) was noted although a mean of 3.1 mg of P.G. was found from the 19 to 25 days of the cycle, after obtaining a zero value on the 17th day. The conclusion that progesterone was being secreted in amounts sufficient to produce P.G., but insufficient to affect the vaginal mucosa seemed unsatisfactory, since crucial evidence was lacking to establish that the isolated material was actually P.G. Two cycles later, simultaneous vaginal smears and daily urine specimens from this patient were studied. As was the case in the previous cycle, the smears showed no evidence of luteal

TABLE II.

Comparison of Gravimetric and Chemical Determination of Pregnandiol Glucuronidate in the Urine of 2 Patients Failing to Show Luteal Activity as Evidenced by Vaginal Smears.

		Pregnandiol Glucuronidate in mg per 24 hrs		
Patient	Day of cycle*	Gravimetric	by reduction	Impurity, %
8	17	1.6	0	100
	18	3.0	0.2	93
	19	3.6	0	100
	20	—	—	—
	21	1.6	0	100
	22	2.0	0	100
	23	—	—	—
	24	4.0	0	100
9	5-12†	13.9	0.5	96
	13	0.4	—	—
	14	0.3	—	—
	15	1.3	—	—
	16	6.7	0	100
	17	1.5	0	100
	18	1.5	0	100
	19	1.5	0	100
	20	0.2	—	—
	21	6.0	0.9	85

*Onset of menstruation—1st day.

†Gravimetric values on 8 successive days were 2.4, 2.7, 2.2, 2.0, 2.5, 0.5, 0.8, and 0.8 mg respectively.

activity. The material isolated and gravimetrically determined as P.G. is clearly not that substance, since it is apparent that 93-100% of this material consists of impurities. This P.G.-like material appears indistinguishable from amorphous Na P.G. by color and solubility properties. The second acetone precipitation used by Venning[4] for final purification precipitates this impurity as well as Na P.G. Similar results were obtained on patient 9, who also failed to exhibit luteal activity as determined by vaginal smears.

Summary. The results presented indicate the necessity for determination of the purity of the P.G. isolated from non-pregnant urine. It has been clearly shown that the modifications reported by Venning[4] do not necessarily remove all impurities from the final product. The implications of these findings, for much of the data in the literature, are clear. In the light of our results, the finding (first reported by Hamblen, *et al.*,[2]) that P.G. may be present in the absence of a secretory endometrium, particularly warrants critical reëxamination.

13546 P

Intrapleural Tuberculosis Following Talc Pleuritis.

EMIL BOGEN, J. J. SINGER AND NATHAN HIATT.

From the Olive View Sanatorium and the Rose Lampert Graff Foundation, Los Angeles, California.

The artificial induction of aseptic pleuritis by the injection of talc into the pleural space has been reported by a number of different workers.[1] This procedure has been recommended for the purpose of producing adhesions which will prevent recurrent spontaneous pneumothorax from collapsing the lung, or may allow a selective collapse, with only an affected portion of the lung collapsed. It has also been advised as a preliminary to operations on the lung, such as drainage of abscesses, cysts or cavities, or before partial or complete amputation of a lung, to diminish the extent and severity of subsequent infection.[2] In view of the frequency of tuberculous involvement of the pleura, it seemed desirable to study the effect of such talc pleuritis on intrapleural tuberculosis before endorsing its use in such conditions.

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[1] Bethune, Norman, *J. Thoracic Surgery*, 1935, **4**, 251.

[2] Singer, J. J., Jones, J. C., and Tragerman, L. J., *J. Thoracic Surgery*, 1941, **10**, 251.

Three rabbits were each given 2 g of talc (U.S.P., Merck), suspended in 5 cc of normal saline solution, injected into the right pleural cavity. Four months later these rabbits, together with 3 other previously untreated animals were each inoculated, in the right pleural cavity, with 0.001 mg of a virulent culture of bovine tubercle bacilli. One month after this infection, X-ray examination showed extensive densities throughout both lungs in the 3 animals which had received the talc, with but little change detectable among the 3 controls. At autopsy, 2 months after infection, the talc-treated rabbits showed extensive nodular caseous tuberculous lesions in both lungs, scattered small caseous nodules in both kidneys, caseous mediastinal lymph nodes and extensive pleural adhesions in the right side. The control animals showed much less extensive lesions, limited mainly to the intercostal and mediastinal lymph nodes, with little or no pulmonary or renal involvement, and no pleural adhesions.

Repetition of this experiment, with different amounts of talc and with different time intervals between the treatment with talc and the injection of tubercle bacilli, on 3 other occasions have given similar results. Further studies of the mechanism of this phenomenon are being carried out. The observations here reported, however, indicate that induced talc pleuritis increases the spread of later intrapleural tuberculous infection, in rabbits, and suggest that it should not be undertaken in man without due consideration of this danger.

TABLE I.
Extent of Tuberculosis in Experimental Animals.

No. of rabbits	Amt talc given intrapleurally g	Interval before infection, wks	Avg amt of tuberculosis				
			Lungs	Kidneys	Lymph nodes	Pleura	Total
4	4	26	+++	+	+	++	7
3	2	16	+++	++	+	++	8
3	1	3	+++	+	+	++	7
3	1	1	+++	+	+	+	6
10	None	Infected controls	++	+	+	0	4
4	1	Uninfected controls	0	0	0	0	0

13547

Inhibition of Gastric and Cecal Peristalsis in Rabbit by $CO_2 + O_2$ Inhalations.

JOHN AUER AND HUGO KRUEGER.

From the Department of Pharmacology, St. Louis University School of Medicine, St. Louis, Mo.

The effect of carbon dioxide gas upon the motility of the gastrointestinal canal has been studied chiefly on excised sections of the gut[1-4] or by the ingestion of water saturated with CO_2 or by the intragastric injection of the gas itself.[5] As far as we know the effect of $CO_2 + O_2$ inhalations upon gastric and cecal motility induced by the intragastric injection of air or by the subcutaneous injection of pilocarpine or physostigmine has not been reported. Furthermore, it may be emphasized that the results to be reported were obtained without opening the peritoneal cavity or the use of balloons but simply by inspection of the intact abdomen.

The method is a slight modification of one described sometime ago:[6] preliminary narcotization to reduce or abolish the inhibitory effects of external stimuli; to accomplish this 150 mg sodium barbital per kilo were injected subcutaneously about 30 minutes before the test; after cutting the abdominal hair, which is readily done with a blunt-pointed scissors curved on the flat, both the cecum and most of the stomach including the preantrum may be easily observed through the intact abdominal wall; the antrum itself is not visible, but its contraction is readily indicated by a bulging of the preantrum. If the stomach was not well-filled, 60-120 cc air were injected into this viscus through a hypodermic needle plunged through the abdominal wall. The gas mixtures were 50% each CO_2 and O_2 delivered from tanks at a rate of 150-300 cc per minute each; this gas mixture was administered to the rabbit through a funnel held at such a distance over the nose or the tracheal tube that the respirations were definitely deepened. All observations were 5 minutes long: before, during and after the gas administration. Gastric and cecal peristalsis was stimulated by the subcutaneous injection of physostigmine sulfate

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[1] Rona, P., and Neukirch, P., *Pflüger's Arch.*, 1912, **146**, 371.

[2] Hooker, D. R., *Am. J. Physiol.*, 1912-13, **31**, 47.

[3] Mansfeld, G., *Pflüger's Arch.*, 1921, **188**, 241.

[4] Guggenheim, M., and Löffler, W., *Biochem. Z.*, 1916, **72**, 322.

[5] Carlson, A. J., *Am. J. Physiol.*, 1913, **32**, 389.

[6] Auer, J., PROC. SOC. EXP. BIOL. AND MED., 1906, **4**, 8.

or pilocarpine hydrochloride in doses of 0.5-1 mg per kilo; occasionally this dose was repeated after 30 minutes.

A few experiments were made on barbitalized, intact rabbits to see whether mere distension of the stomach with air would suffice to bring about good basal peristaltic activity of both stomach and cecum. In 3 rabbits, gastric peristalsis was developed and this was definitely reduced by $CO_2 + O_2$; cecal peristalsis, however, was absent in 2 and only moderate in the remaining rabbit; in this last rabbit, $CO_2 + O_2$ produced a definite decrease. Mere distension of the stomach with air, therefore, did not suffice for our purposes.

Interference with respiration in the normal rabbit by increased salivation after pilocarpine was largely if not entirely abolished by gauze drainage wicks placed in each angle of the mouth and by elevating the foot of the operating board to 35° to facilitate drainage; in the operated animals, a tracheotomy tube prevented this interference. To rule out effects from the central nervous system, the vagi were cut in the neck after tracheotomy, the spinal cord transected at the 4th to the 7th thoracic level, and then the peripheral section of the cord was pithed; the site of transection and the extent of pithing was always verified by autopsy. For all spinal cord operations the barbitalized rabbits were fully anesthetized with ether; after pithing, the ether was stopped and the animal allowed to recover. Biopsies were made on all operated animals and the lungs were found to collapse practically normally on opening the chest and excising them. In numbers of these animals the abdomen was opened at the end of an experiment and the visible effects of $CO_2 + O_2$ inhalations observed: the small arteries of the gut remained bright red and the veins and venules were only slightly darkened. The rabbits weighed from 1500 to 3000 g; both males and females were used, but no female was pregnant; some rabbits were used twice, once without operative interference and then, 5-10 days later, for pithing of the cord; 27 experiments were carried out.

Results. In normal, non-operated rabbits the subcutaneous injection of physostigmine or pilocarpine generally caused good gastric and cecal peristalsis and the inhalation of $CO_2 + O_2$ definitely reduced this peristalsis in strength and rate, though at times only the strength of the waves was reduced, the rate remaining practically unaltered; for example in the stomach, under the influence of CO_2, the visible waves started at the middle line of the abdomen and faded away at the preantrum, while before the CO_2 they started 1 to 2 cm to the left of the middle line, were deeper and progressed through the preantrum.

In the non-operated physostigmine series, 4 out of 7 rabbits showed a definite reduction in *gastric* peristalsis during $CO_2 + O_2$ inhalation; 2 showed no definite peristalsis of the stomach at any time during the experiment; and in one the gas mixture exerted no appreciable effect upon the rate and strength of the gastric waves. All the *ceca* of this series exhibited a definite decrease in the rate and strength of peristaltic and antiperistaltic waves during the gas inhalation.

In the non-operated pilocarpine series of 6 rabbits, comparable effects to those described for physostigmine were obtained. In this series also, one stomach with active peristalsis was refractory to the action of $CO_2 + O_2$.

In the operated physostigmine series (vagotomy, spinal transection and pithing) a striking difference became observable: 5 out of 6 rabbits developed no *gastric* peristalsis at all, while in the 6th rabbit good gastric waves were present but remained practically unaffected by $CO_2 + O_2$ inhalations; *cecal* peristalsis, however, was good and the gas mixture definitely decreased its rate and strength in 5 out of the 6 rabbits.

In the operated pilocarpine series a similar effect to that described above was obtained: 3 out of 5 rabbits showed no visible *gastric* peristalsis throughout the entire experiment, the remaining 2, however, showed good gastric peristalsis that was definitely decreased by $CO_2 + O_2$ inhalation. All rabbits of this series also showed *cecal* peristalsis and this was decreased by $CO_2 + O_2$. In both of these series the stomach had been distended with air about 10 minutes after pithing.

Summary and Conclusion. In the normal rabbit, gastric and cecal peristalsis caused by the subcutaneous injection of physostigmine or pilocarpine (0.5 to 1 mg per kg) is definitely reduced by the inhalation of non-asphyctic doses of $CO_2 + O_2$ gas (50% each).

When the stomach and cecum are deprived of their central innervation by double vagotomy, spinal transection and pithing, both physostigmine and pilocarpine in normally effective doses, become largely ineffective in provoking *gastric* peristalsis; *cecal* peristalsis, however, persists with both drugs in these operated animals and this cecal activity is reduced by $CO_2 + O_2$ inhalation.

As far as the *cecum* is concerned, the experimental findings indicate clearly that $CO_2 + O_2$ inhalations exert a *peripheral,* inhibitory action upon cecal peristalsis produced by the subcutaneous injection of physostigmine or pilocarpine in the pithed rabbit, for cecal peristalsis developed in the pithed rabbits after the injection of the drugs and this cecal peristalsis was definitely reduced by the gas inhalation.

Furthermore, it may be concluded that vascular shock in these pithed animals did not prevent the occurrence of cecal peristalsis after physostigmine and pilocarpine nor did shock prevent the inhibitory action of $CO_2 + O_2$ inhalations upon this cecal peristalsis.

With regard to the *stomach*, however, the experimental data do not permit a similar conclusion, for normally effective doses of the drugs generally caused little or no apparent gastric peristalsis in the pithed animals, thus preventing a study of the inhibitory action of the gas mixture. This difference between the cecum and stomach might possibly be caused by a difference in sensitivity to the drugs employed, the cecum responding to smaller doses of the drugs than the stomach, for the drugs were always injected after pithing when the blood pressure was low and the speed of circulation probably decreased. In addition, this low blood pressure itself might be assumed to be a factor in preventing or reducing *gastric* peristalsis, though low blood pressure is definitely not an important factor in *cecal* peristalsis under the experimental conditions described.

It may perhaps be suggested that inhalations of $CO_2 + O_2$ mixtures might be of some value in the human subject under conditions of gastric and perhaps colonic unrest.

13548

Effect of Stimulation of Autonomic Nerves on Intrahepatic Circulation of Blood in Intact Animal.*

Khalil G. Wakim. (Introduced by F. C. Mann.)
From the Mayo Foundation, Rochester, Minn.

The influence of the sympathetic and parasympathetic nerves on the intrahepatic vascular system has been deduced mainly from indirect evidence based on data obtained from plethysmographic changes in volume of the liver and from changes in hepatic inflow or outflow, as well as from variations in portal pressure during stimulation of the nerves. The object of this study was to observe directly, by transillumination of the intact liver in the anesthetized animal the effect of stimulation of the sympathetic and parasympathetic nerves on the intrahepatic vessels and to determine whether or not the intrahepatic vascular system possesses an active vasoconstrictor or an active vasodilator mechanism or both.

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The literature on the subject discloses a number of different views held by investigators who employed various methods for determining the effect of stimulation of the autonomic nerves on the vascular ramifications within the liver. Bayliss and Starling[1] obtained a rise in portal pressure by electric stimulation of the splanchnic nerves. Cavazzani and Manca[2] produced constriction of the portal radicles by asphyxia after sectioning the vagus nerves, but not after sectioning the splanchnic nerves. They stated that the vagus nerves cause direct and not reflex dilation of the portal radicles within the liver. In another publication they reported that the intrahepatic branches of the hepatic artery constrict during vagal stimulation and dilate during stimulation of the celiac plexus. François-Franck and Hallion[3] obtained a decrease of hepatic volume and a rise of portal pressure in the dog when the splanchnic nerves were stimulated. The same results were obtained after ligation of the hepatic artery. Thompson[4] reported immediate decrease of hepatic volume during stimulation of the splanchnic nerves. Schmid[5] obtained a decrease of inflow through the portal vein and a rise of portal pressure by stimulation of either the splanchnic nerves or the hepatic plexus. Burton-Opitz[6] produced total cessation of inflow of blood through the hepatic artery by tetanic stimulation of the whole hepatic plexus. In another publication he concluded that stimulation of the distal end of the hepatic plexus during perfusion as well as in the normal portal circulation proved that the portal radicles have vasomotor fibres from the hepatic plexus. Neubauer[7] recorded an increase of hepatic volume during stimulation of the central end of the vagus and also during stimulation of the central end of the splanchnic nerves. Stimulation of the peripheral end of the vagus caused a decrease of hepatic volume which was attributed to constriction of the intrahepatic vessels. Macleod and Pearce[8] expressed the opinion that stimulation of the hepatic plexus causes a greater constriction of the branches of the hepatic artery than of those of the portal vein. Edmunds[9] explained the increase of hepatic volume during splanchnic stimulation as due to the constriction of the muscle fibers around the sublobular veins. He also stated that vasomotor nerves are present on the portal branches in the liver. Carnot, Gayet and Merklen[10] obtained a rise of portal pressure during stimulation of the peripheral end of the vagus or of the splanchnic nerves. This rise was attributed to constriction of the hepatic vessels. Griffith and Emery[11] obtained a decrease of hepatic volume during stimulation of the preganglionic or postganglionic nerve fibers which supply the liver. Since stimulation of the peripheral end of the vagus did not cause any change of hepatic volume, they concluded that the vagus nerves do not carry vasomotor fibers to the liver. Bauer, Dale, Poulsson and Richards[12] produced a decrease of hepatic volume, increase of outflow, increase of portal flow and arteriolar constriction within the liver by stimulation of the hepatic plexus or of the right or left splanchnic nerves, while stimulation of the vagus did not give any perceptible change. Eckardt[13] concluded that during splanchnic stimulation an outpouring of blood from the liver takes place and that the amount of blood leaving the liver is greater than that entering it. McMichael[14] stated that there was no evidence indicating parasympathetic dilator action on the portal or hepatic venules.

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[8] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1914, **35**, 87.

Methods. A modification of the quartz-rod transillumination technic as described by Knisely[15] was employed in this study on anesthetized frogs, and albino rats. The frogs were studied at room temperature, but the rats were kept at body temperature during observation by the use of a constant temperature platform. The hepatic plexus was dissected out carefully and, with the vessels of the porta hepatis, was placed in the trough of specially made shielded electrodes, and stimulated by tetanizing current when desired. In the rat, the vagi were separated from the esophagus at its exit from the esophageal hiatus in the diaphragm and were ligated tightly and placed in the trough of the electrodes distad to the ligature.

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[13] Eckardt, P., *Arch. f. d. ges. Physiol.*, 1935, **236**, 361.

[14] McMichael, John, *Quart. J. Exp. Physiol.*, 1937, **27**, 73.

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Results. Observations on the Amphibian Liver. Stimulation of hepatic plexus. Stimulation of the nerve plexuses in the porta hepatis and around the hepatic artery by weak or strong tetanizing current caused constriction of the active sinusoids, some of which contracted. For instance, fourteen active sinusoids were counted in a region immediately before stimulation. During stimulation only six of the fourteen sinusoids remained patent but these were constricted to such an extent that the corpuscles were compressed into single file as they coursed through the narrow lumen. The other 8 sinusoids contracted to disappearance during stimulation. The constriction of the sinusoids appeared within two seconds after the beginning of stimulation and lasted for 4 to 5 seconds after discontinuance of the stimulation. On several occasions when an arteriovenous anastomosis was present in the field under observation, the anastomosis disappeared during stimulation and continuity between the two vessels no longer could be seen.

Effect of Pithing. Pithing the animal, or any severe damage to the central nervous system, led to a marked reduction in the number of active sinusoids. In fact, immediately after pithing, inactivity in the lobules becomes so extensive that one would be inclined to believe the animal is dead were it not for the continuous activity of a regularly beating heart. This inactivity may result in either a constrictor effect, leading to very narrow sinusoids containing hardly any corpuscles, or more commonly in stasis with the sinusoids packed full of corpuscles lying motionless in their lumina. However, after about 15 minutes, the circulation begins to recover in the inactive lobules and one gradually can observe the same type of rhythmic activity alternating asynchronously in the various lobules. Many more inactive sinusoids packed full of erythrocytes are seen in the liver of a pithed frog than in an intact anesthetized one.

Observations on the Mammalian Liver. Stimulation of hepatic plexus. Stimulation of the plexus of nerves in the porta hepatis and around the hepatic artery by tetanizing current produced moderate blanching of the region under observation and constriction of the sinusoids. When weak current was used, it took more than 5 seconds of latency before the sinusoids contracted and blanching was observed. After cessation of the stimulus, more than 10 seconds passed before the sinusoids resumed activity. In some lobules several sinusoids that were very active prior to strong stimulation contracted to disappearance and remained so for several seconds after the current had been discontinued. Arterial sinusoids were constricted more markedly than the venous ones during the stimulation. The arteriovenous anastomoses behaved in the same manner as the sinusoids toward electric stimulation of the hepatic plexus. They constricted to disappearance, and continuity between the ramifications of the hepatic artery and the portal vein no longer could be observed. It was noted that comparatively inactive lobules, in which very few sinusoids were patent prior to stimulation, became very active when the current was discontinued and hardly any sinusoids could be seen in phases of inactivity in such lobules for a period of more than twenty minutes thereafter.

Stimulation of the Vagi. Stimulation of both vagus nerves, at their exit with the esophagus from the esophageal hiatus of the diaphragm, by various strengths of tetanizing current, did not cause any perceptible changes in the circulation of the liver.

Summary. The effects of electric stimulation of the hepatic plexus and of the vagus nerves on the intrahepatic circulation of blood in the intact animal were studied by means of the quartz-rod transillumination technic. Stimulation of the vagus nerves did not have any perceptible effect on the intrahepatic vessels. Stimulation of the hepatic plexus caused constriction of the intrahepatic vessels.